CHARLES UNIVERSITY IN PRAGUE

Faculty of Pharmacy Hradec Králové

Department of Pharmacology and Toxicology

DIPLOMA THESIS

2013 Lenka Burgetová

KARLOVA UNIVERZITA V PRAZE

Farmaceutická fakulta v Hradci Králové

Katedra farmakologie a toxikologie

CARDIFF UNIVERSITY

School of Pharmacy and Pharmaceutical Sciences

Breast Cancer Molecular Pharmacology Group

Studium role zinkového transportéru ZIP 6 a STAT3 v mitóze

DIPLOMOVÁ PRÁCE

Školitel: PharmDr. Martina Čečková PhD.

Školitel specialista: Dr. Kathryn Taylor PhD.

Erasmus Project

15.květen – 10.září 2012

Lenka Burgetová

CHARLES UNIVERSITY IN PRAGUE

Faculty of Pharmacy Hradec Králové

Department of Pharmacology and Toxicology

CARDIFF UNIVERSITY

School of Pharmacy and Pharmaceutical Sciences

Breast Cancer Molecular Pharmacology Group

Investigating the role of zinc transporter ZIP 6 and STAT3 in mitosis

DIPLOMA THESIS

Supervisor: PharmDr. Martina Čečková PhD.

Specialized supervisor: Dr. Kathryn Taylor PhD.

Erasmus Project

May 15th – September 10th 2012

Lenka Burgetová

DECLARATION

Prohlašuji, že tato práce je mým původním autorským dílem.Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Tato práce nebyla použita k získání jiného či stejného titulu.

I declare that this thesis is the result of my own investigation, except where otherwise stated. All literature and other resources I used while processing the thesis are listed in the bibliography and properly cited.

Signed ..... (Candidate)

Dated .....

Acknowledgements

I would like to thank Dr. Kathryn Taylor PhD. for her support, guidance, patience and for giving me the opportunity to join the Breast Cancer Molecular Pharmacology Group at Cardiff University.

I am thankful to Lindy Goddard and Anna Burt for their assistance and support.

My very sincere thanks to PharmDr. Martina Čečková PhD. for helping me with writing this thesis.

Last but not least, I would like to thank my parents, Zdeňka Burgetová and Pavel Burget for their love and continuous support – both spiritually and materially.

Abstrakt

Univerzita Karlova v Praze

Farmaceutická fakulta v Hradci Králové

Katedra farmakologie a toxikologie

Studentka: Lenka Burgetová

Školitel: PharmDr. Martina Čečková PhD.

Školitel specialista: Dr. Kathryn Taylor PhD.

Název diplomové práce: Studium role zinkového transportéru ZIP 6 a STAT3 v mitóze

Bylo prokázáno, že STAT3 (přenašeč signálu a aktivátor transkripce 3) se podílí na vzniku rakoviny. ZIP6 je cílem tohoto transkripčního faktoru. Větší část dosavadního výzkumu byla zaměřena jen na aktivaci STAT3 po fosforylaci tyrosinu, zatímco fosforylace serinu zůstala relativně neprozkoumána. Domníváme se, že fosforylace serinu je aktivována v průběhu mitózy tamoxifen-rezistentních prsních nádorových buněk (TamR), a že zinkový transportér ZIP6 a na serinu fosforylovaný STAT3 jsou zahrnuty v zinkem zprostředkovaném mechanismu mitózy. Při použití nocodazolu pro navození zástavy mitózy, jsme pozorovali snížení exprese na tyrosinu fosforylovaného STAT3, zatímco exprese STAT3 fosforylovaného na serinu byla u mitotických buněk zvýšena. Přidání zinku mělo za následek vyrušení účinku nocodazolu, obnovení buněčného cyklu a proteolytické štěpení STAT3, což svědčí pro nový mechanizmus úniku z mitózy. Dále jsme objevili, že ZIP6 je v průběhu mitózy štěpen na N-terminálním úseku a přemístěn z endoplazmatického retikula na plazmatickou membránu, kde přenáší zinek dovnitř do buňky.

Abstract

Charles University in Prague

Faculty of Pharmacy in Hradec Králové

Department of Pharmacology and Toxicology

Student: Lenka Burgetová

Supervisor: PharmDr. Martina Čečková PhD.

Specialized supervisor: Dr. Kathryn Taylor PhD.

Title of diploma thesis: Investigating the role of zinc transporter ZIP 6 and STAT3 in mitosis

It has been shown that STAT3 (signal transducer and activator of transcription 3) plays a role in the development of cancer. ZIP6 is the downstream target of this transcription factor. Previous research has focused mainly on the activation of STAT3 by tyrosine phosphorylation, while the effect of phosphorylation at a second site, serine 727, remained relatively uninvestigated. In this study, it is proposed that serine-phosphorylated STAT3 is activated throughout mitosis in tamoxifen-resistant (TamR) breast cancer cells and that zinc transporter ZIP6 and serine-phosphorylated STAT3 are involved in a zinc-mediated mitotic mechanism. After using nocodazole to induce mitotic arrest, the expression of tyrosine-phosphorylated STAT3 protein was observed to be reduced while the expression of serine-phosphorylated STAT3 was increased. Zinc supplementation after nocodazole treatment appeared to push cells through mitotic-arrest and cause proteolytic cleavage of STAT3 suggesting a novel mechanism for mitotic exit. Furthermore, we discovered that ZIP6 was processed by N-terminal cleavage in a mitotic related manner, which caused relocation from the endoplasmic reticulum to the plasma membrane, where it has been shown to influx zinc.

Contents

1. List of abbreviations

Table 1: Abbreviations

Abbreviation	Meaning
APS	Ammonium Persulphate
BRCA	Breast cancer gene
BSA	Bovine Serum Albumin
c-erbB2	Protein product of HER-2 oncogene
CK2	Casein kinase 2
CON	Untreated cells with nocodazole
DAPI	4,6-diamino-2-phenylindole
DPBS	Dulbeccos' phosphate buffered saline
DTPA	Diethylenetriaminepentaacetic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene Glycol Tetraacetic Acid
ER	Oestrogen Receptor
FCS	Foetal Calf Serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
MAPKs	Mitogen-activated Protein Kinases
MCF-7	Breast cancer cells
mRNA	messenger Ribonucleic Acid
NOC	Cells treated with nocodazole
P	Pyrithione
PBS	Phosphate Buffered Saline
PMSF	Phenylmethylsulfonyl Fluoride
PP2A	Protein Phosphatase 2A
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis
SE	Standard Error
SERMs	Selective Receptor Modulators
SFCS	Steroid-depleted Foetal Calf Serum
Src	Tyrosine-protein kinase
STAT3	Signal Transducer and Activator of Transcription 3
TAD	Transactivation Domain
Tam	Tamoxifen
TamR	Tamoxifen-resistant breast cancer cells
TBS	Tris-Buffered Saline
TEMED	N,N,N´,N´-tetramethylethylenediamine
TPEN	N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine
WBR	Western blocking reagent

2. Introduction

Breast cancer is the most common cancer and the leading cause of cancer death in women worldwide (Jemal et al. 2011). It is recognised that two-thirds of diagnosed breast cancers express oestrogen receptor α (ERα) and are likely to be hormone-responsive (Sainsbury, 2004). As oestrogen and the ER play an important role, the first line of therapy for ERα-positive breast cancers usually involves the use of selective receptor modulators (SERMs), such as tamoxifen, although in post-menopausal women aromatase inhibitors may also be used. Most tumours however, eventually become resistant to tamoxifen and alternative treatments are required (Sainsbury, 2004). It has been shown, that tamoxifen resistant tumours possess higher levels of intracellular zinc due to overexpression of zinc transporters (Taylor et al. 2008). A tamoxifen-resistant MCF-7 breast cancer cell line (TamR), developed at the former Tenovus Centre for Cancer Research, Cardiff University, has also been demonstrated to have increased intracellular zinc compared with the hormone responsive parent cell line (Taylor et al. 2008). For this reason, the TamR cell line is an ideal model for studying the role of zinc in breast cancer. Although a lot is known about zinc, the part played in hormone-resistant breast cancer is not well understood. Zinc is unable to passively diffuse through cell membranes therefore it needs to be transported into intracellular compartments. There are two main families of zinc transporters which transport Zn^{2+} across membranes in opposing directions. The ZnT family is responsible for zinc efflux from the cytoplasm into various intracellular compartments or across the plasma membrane (Palmiter and Huang, 2004) while the ZIP family is responsible for zinc influx into cells or from vesicles or organelles (Kambe, 2011). Among ZIP transporters, nine belong to the LIV-1 subfamily (Taylor and Nicholson, 2003). For this project, the LIV-1 subfamily was considered the most interesting, since several members have been associated with breast cancer (Manning et al. 1995). One member, in particular, of the LIV-1 subfamily, ZIP6 (or SLC39A6), is the downstream target of the transcription factor STAT3 (signal transducer and activator of trancription 3) in zebrafish embryos (Yamashita et al. 2004). STATs control cellular growth, differentiation, development and survival (Bowman et al. 2000) and importantly have been shown to be constitutively activated in breast carcinoma cell lines but not in normal breast epithelial cells (Garcia et al. 2001). Furthermore, inhibition of this constitutive activation resulted in growth suppression and induction of cell death. A recent study has also shown that

zinc directly binds the α-helical secondary structure of STAT3 and suppresses activation on Tyr705 (Kitabayashi et al. 2010). While less is known about pSTAT3(Ser727) activation, other reports have shown that phosphorylation on serine 727 may be important for transcription (Wen et al. 1995, Zhang et al. 1995, Ng and Cantrell, 1997) and interestingly, observations in our laboratory indicated that pSTAT3(Ser727) was activated throughout mitosis (Taylor, unpublished). The aim of the current study, therefore, was to further investigate the known association of ZIP6 and STAT3 with regard to mitosis and in relation to phosphorylation of both Tyr705 and Ser727, in the TamR breast cancer cell model. It is suggested that both ZIP6 and STAT3 are involved in mitosis and the study of their role in this mechanism may lead to the identification of potential targets for new pharmacological treatment.

3. Background

3.1 Breast cancer

Cancer is not a single disease but a group of diseases characterized by loss of normal cell regulation leading to uncontrolled cell division and subsequent metastasis of cells to other tissues or organs (American Cancer Society). Breast cancer is the most common type of the disease and the leading cause of cancer death in women worldwide (Jemal et al. 2011), accounting for 226 870 of the new cancer cases and 39 510 of the cancer deaths in the United Stated in 2012 (Siegel et al. 2012). It is recognised that two-thirds of diagnosed breast cancers express oestrogen receptor α (ERα) and are, therefore, likely to be hormone-responsive (Sainsbury, 2004). Because of the important role of oestrogen and the ER in breast cancer, the first line of therapy is to block binding of oestradiol to ER and/or to lower the level of oestrogen for the control and the prevention of the disease. This may be done in a number of ways. One approach was to surgically remove the ovaries (in pre-menopausal women) or the adrenal glands (in post-menopausal women) to prevent the production of androgens along with chemotherapy with its detrimental side effects. The benefit of ovarian ablation was first appreciated by the Scottish physician George Beatson in 1896 (Beatson, 1896) and the benefit of removing adrenal glands was first achieved by the American physician and physiologist Charles Huggins in 1952 (Huggins, 1966). With the development of new drugs, it is now more common to use endocrine therapy for oestrogen receptor positive patients. Selective receptor modulators (SERMs) such as tamoxifen are the most common therapy for ERα-positive breast cancer, although in post-menopausal disease aromatase inhibitors are also used. Aromatase inhibitors, such as exemestane and anastrozole, prevent the synthesis of oestrogen. Tamoxifen, on the other hand, competes with the natural hormone, oestradiol, for binding to the ligand binding domain of oestrogen receptor and is also a partial agonist. It blocks oestrogenic growth signals in breast tissue, while maintaining oestrogenic responses in bone tissue (Nichols, 2007) and is associated with an increase in the incidence of endometrial cancer (Fisher et al. 1996) and of thromboembolic disease (Fisher et al. 1996, Jaiyesimi et al. 1995). Moreover, and of especial importance, most tumours eventually become resistant to tamoxifen and alternative treatments are required (Sainsbury, 2004). For this reason tamoxifen

resistant breast cancer cell lines are frequently used in breast cancer research including that undertaken in this investigation.

3.2 Signs and symptoms of breast cancer

Breast cancer is without symptoms when the tumour is small and most treatable but when the tumour has grown to a size that can be felt, a common physical sign is a painless lump (American Cancer Society). Breast cancer can spread to underarm lymph nodes and cause swelling or a lump, even before the original breast tumour is large enough to be felt. Breast pain or heaviness, persistent changes to the breast, such as swelling, thickening, or redness of the breast skin, and nipple abnormalities such as spontaneous discharge (especially if bloody), erosion, inversion or tenderness are less common. It is important to note that pain does not indicate the presence or the absence of breast cancer (American cancer society).

3.3 Risk factors of breast cancer

a) Unalterable risk factors

1. Sex

The main risk factor for developing breast cancer is female gender with 100 times higher incidence in female than in male (Hulka and Moorman, 2001).

2. Age

The second strongest risk factor is age. The older the woman, the higher her risk (Cancer research UK, Breast cancer risk factors). Breast cancer rates start to increase in the late 20s (Parkin et al. 1997).

3. Genetic risk factors

Between 5% and 10% of breast cancer cases result from an inherited predisposition (Casey, 1997). Women with a family history of breast cancer, especially in a first-degree relative are at increased risk of developing breast cancer and the risk is higher if

more than one first-degree relative developed breast cancer. Compared to women without a family history, risk of breast cancer is nearly twice as high for women with one first-degree female relative who has been diagnosed, nearly three times higher for women with two relatives, and nearly four times higher for women with three or more relatives (Collaborative Group on Hormonal Factors in Breast Cancer, 2001). A number of genes have been identified as increasing the likelihood of developing familial breast cancer (Schippinger et al. 2006). With hereditary susceptibility to breast cancer are associated mutations in BRCA1 and BRCA2 (Miki et al. 1994, Wooster et al. 1995). BRCA1 mutations are three times more prevalent than BRCA2 mutations (Shih et al. 2002). Patients with mutations in the BRCA1 and BRCA2 genes have a 40% to 80% chance of developing breast cancer (Claus et al. 1991, Easton et al. 1995, Schubert et al. 1997). However, the majority of multiple case breast cancer families do not segregate mutations in these genes. Subsequent genetic linkage studies have failed to identify further major breast cancer genes (Smith et al. 2006). These observations have led to the proposal that breast cancer susceptibility is largely polygenic (Antoniou et al. 2004).

4. Other unalterable risk factors

The breast cancer incidence and mortality rates vary greatly around the world. In general, the rates are lowest in less developed countries of the Far East, South Africa and Africa, Central America, South America and highest in the more developed countries of North America, Europe, Australia, New Zealand (Coleman et al. 1993, Kamangar et al. 2006). Another strong risk factor is denser breast tissue (extensive percent mammographic density). Breast tissue is composed of fat, connective tissue and epithelial tissue. Breasts with a high proportion of fatty tissue are less dense (Boyd et al. 2011, McCormack and dos Santos Silva, 2006). The longer a woman menstruates, the higher her lifetime exposure to the ovarian hormones oestrogen and progesterone and this is associated with a higher risk of breast cancer (Breastcancer.org, Menstrual History). This can be through early age at menarche (Kelsey et al. 1993) with each one year delay in onset leading to a 5% decrease in risk of developing breast cancer later in life (Key et al. 2001) or having menopause older than fifty five (Trichopoulos et al. 1972).

b) Hormonal influence

Reproductive hormones increase the risk of breast cancer. They stimulate breast cell division. The mitotic rate of breast cells is higher during the luteal phase of the menstrual cycle than during the follicular phase. The risk of breast cancer could be reduced by delaying the onset of regular menstrual cycles and by minimizing the therapeutic use of oestrogens, and possibly of progestagens, in post-menopausal women (Key and Pike, 1988).

There are many hormonally relevant factors such as bilateral oophorectomy. The earlier in life that the ovaries are removed, the greater the risk reduction (Trichopoulos et al. 1972). Increased bone mineral density, as a marker of higher integrated oestrogen exposure over time in postmenopausal women (Kuller et al. 1997). Since oestrogens are known to help retain bone mass, the inference is made that bone density and breast cancer risk are correlated as a function of the amount of oestrogen available to the target tissue (Hulka and Moorman, 2001).

c) Lifestyle-related factors

It is believed that lifestyle or environmental factors increase the likelihood of developing breast cancer. One of them is excessive alcohol use (Key et al. 2006), there is a dose-response association between alcohol consumption and risk of breast cancer (Schatzkin and Longnecker, 1994). Obesity in post-menopausal women is thought to act through a hormonal mechanism. The oestrone levels of obese women after menopause are about 40% higher than the levels of non-obese women (Cauley et al. 1989). Less well understood is the observation that obesity may be protective for premenopausal disease (Hulka and Moorman, 2001). Recent oral contraceptive use (Collaborative Group on Hormonal Factors in Breast Cancer, 1996) and hormone replacement therapy (Collaborative Group on Hormonal Factors in Breast Cancer, 1997) have been found as negative risk factors to developing breast cancer. Furthermore, both active smoking and passive smoking increase the risk of breast cancer (Palmer et al. 1991). Cigarette smokers have significantly higher levels of androstenedione than non-smokers, although there is little difference in serum oestrogens (Cauley et al. 1989). Positive lifestyle-related factors, which can lower the risk of breast cancer include, having many

pregnancies (Ma et al. 2006) and becoming pregnant at a young age (MacMahon et al. 1970), breast-feeding (Collaborative Group on Hormonal Factors in Breast Cancer, 2002) and physical activity, with more active women having lower levels of oestrone (Cauley et al. 1989).

d) Others

Other risk factors are exposure to high-dose ionizing radiation to the chest, such as that delivered to children to reduce the size of the thymus gland or repeated fluoroscopies used during lung-collapse therapy for tuberculosis (Hildreth et al. 1989, Miller et al. 1989). Also implicated are low vitamin D levels (Crew, 2013), prolonged periods of night shift work (Leonardi et al. 2012), exposure to diethylstilbestrol (Breastcancer.org, DES (diethylstilbestrol) Exposure) and certain chemicals in cosmetics (Darbre, 2003, Breastcancer.org, Exposure to Chemicals in Cosmetics).

3.4 Tamoxifen resistance

Antioestrogens such as tamoxifen are the mainstay of treatment for breast cancer. Tamoxifen, which is an oestrogen receptor antagonist was developed over 30 years ago and is used as therapy in pre- and post-menopausal women. Many breast cancers are stimulated to grow by the female sex hormones oestrogen and progesterone. These breast cancers are called hormone sensitive. 70% of all breast cancer cases are oestrogen-receptor positive (Berger et al. 2012). This means that these breast cancer cells have oestrogen receptors, which bind oestrogen, stimulating the cell to divide and grow. Tamoxifen blocks the oestrogen receptor and therefore lowers the risk of breast cancer coming back after surgery and helps to reduce the risk of cancer in the other breast by 40% (Cancer research UK, Types of breast cancer hormone therapy). This therapy benefits approximately 50% of patients (Knowlden et al. 2003) but tumours often acquire resistance, leading to cancer recurrence. Resistance to anti-hormonal drugs is becoming an increasing problem. This is usually the result of cells acquiring the ability to utilize alternative growth factor signalling pathways (Knowlden et al. 2003), which can lead to cancer regrowth, commonly with an altered and more aggressive phenotype (Hiscox et al. 2004). The mechanism of the Tam-resistance is not exactly

known. Both epidermal growth factor receptor and c-erbB2 mRNA and protein expression are increased in TamR compared with wild-type MCF-7 cells (Knowlden et al. 2003). Interestingly, it has been shown that TamR cells have increased levels of intracellular zinc, due to overexpression of zinc transporters, compared with their hormone-responsive counterparts (Taylor et al. 2008) but the role of zinc in tamoxifen-resistance is not well understood. This makes TamRs an ideal cell line for investigating the role of zinc in breast cancer.

3.5 Zinc and breast cancer

Zinc is a regulator of a number of signalling pathways in both cell division and cell death and has an important role to play in ensuring normal tissue homeostasis (Zalewski, 2011). At least 2800 human proteins are potentially zinc-binding, comprising 10% of the human proteome (Andreini, 2006). A large and diverse number of zinc metalloenzymes and proteins have been recognized to participate in the metabolism of proteins, nucleic acids, carbohydrates, and lipids (Vallee and Falchuk, 1993). Zinc has been reported to play an essential role in the cell cycle (Fujioka and Liberman, 1964). When zinc becomes limited by treatment with N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), an intracellular zinc chelator, a decrease in cell proliferation is observed in a cell line derived from a pheochromocytoma of the rat adrenal medulla (PC12), highlighting zinc importance in the cell cycle (Li and Maret, 2009). Many studies have examined the relationship between zinc in serum or plasma with breast cancer. Reported concentrations of plasma zinc in breast cancer patients have been contradictory. A number of studies have reported decreased serum zinc in breast cancer patients (Memon et al. 2007, Yucel et al. 1994, Gupta et al. 1991), whereas others showed increased levels of zinc in the serum (Cavallo et al. 1991) or no change (Piccinini et al. 1996). Interestingly, when measuring the actual zinc content in breast cancer tissue, all results seemed to be consistent with increased levels in cancer tissue compared to normal breast tissue (Santoliquido et al. 1976). It has been shown to be increased by 72% in breast tumours compared to normal breast tissue (Margalioth et al. 1983). These results are reinforced by a model of breast cancer in the rat, induced by N-methyl-N-nitrosourea, displaying an increase of zinc in breast tumours irrespective of the zinc intake (Woo and Xu, 2002). An important component of zinc action in cells is

the ability to inhibit protein tyrosine phosphatase activity (Haase and Maret, 2003), resulting in activation of mitogen-activated protein kinases (MAPKs), as well as the tyrosine kinases Src and epidermal growth factor receptor (EGFR), which rely on dephosphorylation by phosphatases for their inactivation (Wu et al. 1999, Hansson, 1996, Sato et al. 1995)

3.6 Zinc transporters

Zinc is unable to passively diffuse through cell membranes therefore it needs to be transported into intracellular compartments. There are two main families of Zn^{2+} transporters which transport zinc across membranes in opposing directions. These transporters all have multiple domains (*Figure 1)*, and are encoded by two solute-linked carrier gene families. The ZnT family of zinc efflux transporters (termed SLC30A, solute-linked carrier 30) transport zinc out of the cytosol and the ZIP (Zrt- and Irt- like proteins) family of zinc influx transporters (termed SLC39A, solute-linked carrier 39) transport zinc into the cytosol (Liuzzi and Cousins, 2004). The functions and localisation of particular zinc transporters are illustrated in *Figure 2*.

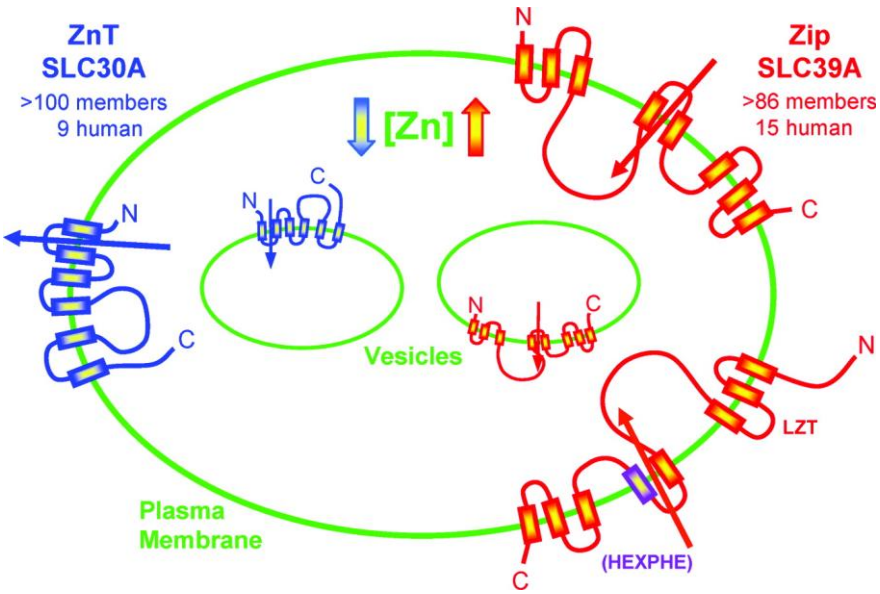

Figure 1: A generalized cell shows the location of the ZIP and ZnT transporter proteins. The ZIP proteins have eight transmembrane domains. The metalloprotease sequence (HEXPHE) of the LIV-1 subfamily (LZT) is shown. The ZnT proteins have six transmembrane domains (adopted from Liuzzi and Cousins, 2004).

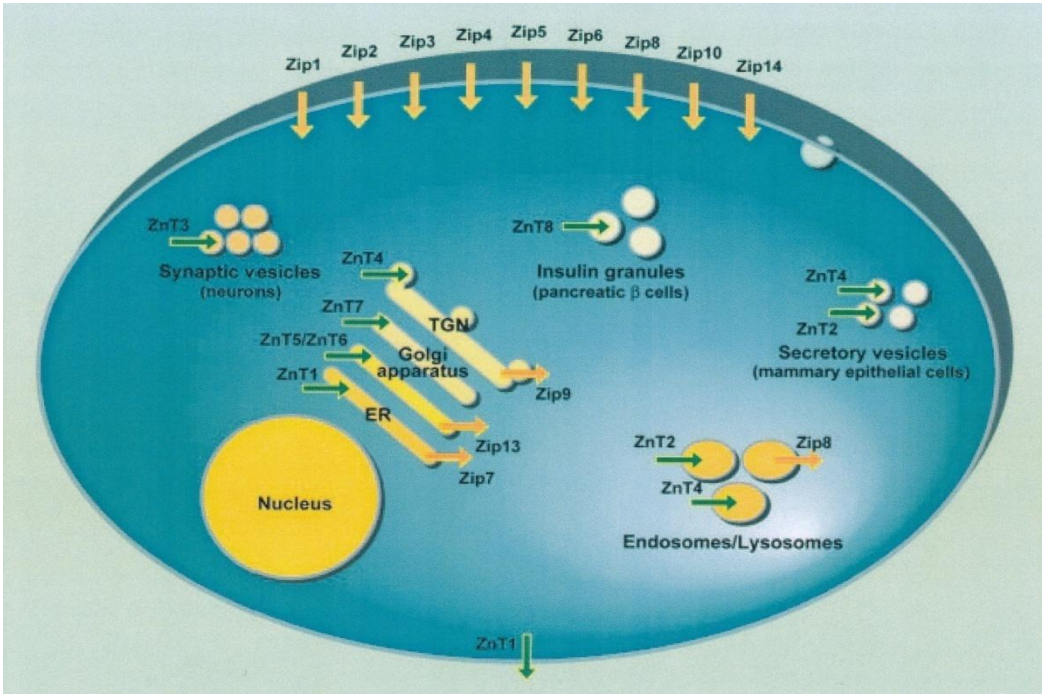

Figure 2: Localisation and function of zinc transporters in the cell. Green and yellow arrows indicate the direction of zinc mobilization by zinc transporters. The ZnT proteins generate zinc efflux out of cells or into vesicles or organelles. The ZIP proteins generate zinc influx into cells or from vesicles or organelles (adopted from Kambe, 2011*).*

3.6.1 ZnT family

The ZnT family consists of ten members (ZnT1-10) and is responsible for the zinc efflux from the cytoplasm either into various intracellular compartments or across the plasma membrane (Palmiter and Huang, 2004). Most of them are predicted to have six transmembrane domains (Kambe, 2011). Both N and C termini are on the inner side of the membrane. Most ZnT proteins have a long intracellular loop with a variable number of histidine residues (Seve et al. 2004). These loops should bind zinc and have been implicated as ion-binding domains (Murgia et al. 1999). ZnT proteins are mainly localized on intracellular compartments like endosomes or Golgi apparatus (Palmiter and Findley, 1995).

3.6.2 ZIP family

The ZIP (Zrt-, Irt-like Protein) family is named after the yeast Zrt1 protein and the Arabidopsis Irt1 protein, the first identified members of this family (Eide, 2006). All ZIP transporters are predicted to consist of eight transmembrane domains with conserved histidine residues (Kambe, 2011). The ZIP family consists of at least 86 members and is divided into four subfamilies (Gaither and Eide, 2001). ZIP subfamily I (mainly fungal and plant sequences), gufA subfamily (is related to the gufA gene of Myxococcus Xanthus), ZIPsubfamily II (mammalian, nematode and insect genes) (Guerinot, 2000) and the LIV-1 subfamily (Taylor and Nicholson, 2003).

The LIV-1 subfamily currently comprises nine members also called LZT (Taylor and Nicholson, 2003). They are situated on the plasma membrane and endoplasmic reticulum and have the ability to transport zinc into cells. For this project the LIV-1 subfamily was thought to be of the highest interest because several members have been associated with breast cancer and LIV-1, shown to be an oestrogen-regulated gene, has also been implicated in breast cancer metastasis (Manning et al. 1995). All family members share considerable homology, as each member consists of eight transmembrane domains with conserved histidine residues both intracellularly and on the N-terminus and extracellular loop (*Figure 3*). In addition, they all have a unique HEXPHE motif in the transmembrane domain V and have a very long N terminus.

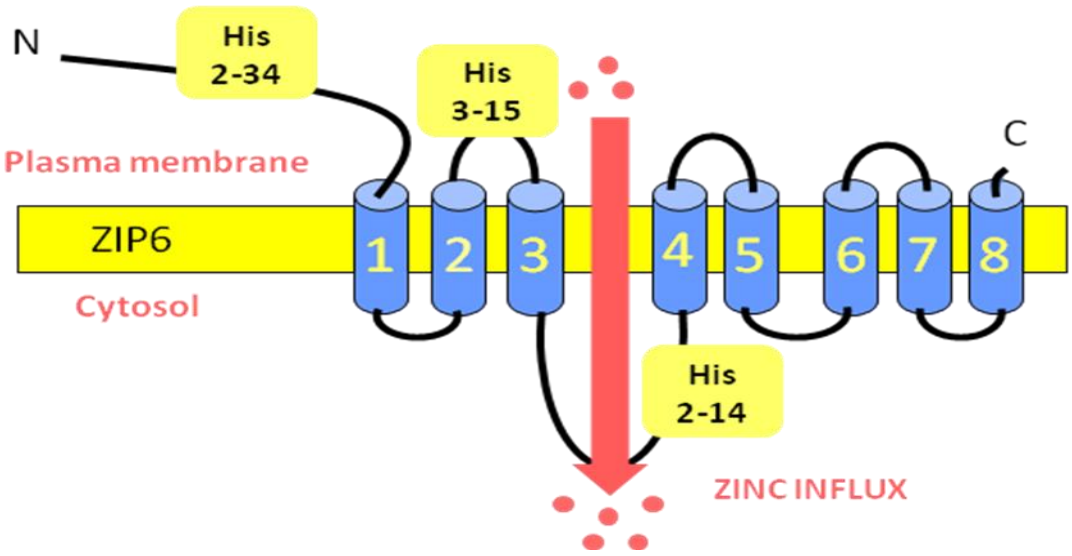

Figure 3: Schematic representation of LIV-1 secondary structure. The three histidine rich regions that are unique to this subfamily are shown in yellow (adopted from Taylor el al. 2003).

3.7 ZIP7-mediated intracellular zinc transport in TamR cells

The cellular location of ZIP7 is unique. Most of the ZIP transporters are present on the plasma membrane. ZIP7 however, has been shown to be located on the endoplasmic reticulum and plays a role in mobilizing intracellular zinc. Various studies have found that zinc levels are increased in human breast cancer cells, leading to increased cell cycling (Santoliquido et al. 1976, Margalioth et al. 1983, Mulay et al. 1971). Zinc plays a part in the development of tamoxifen-resistant breast cancer and in fact, TamR cells have increased ZIP7 and zinc expression. ZIP7 is essential for the zinc-induced inhibition of phosphatases, which leads to activation of growth factor receptors (Taylor et al. 2008). Removal of ZIP7 destroys activation of epithelial growth factor receptor/IGF-I receptor/Src signaling by reducing intracellular zinc levels. There is a significant elevation in growth rate in zinc-treated TamR cells in comparison with untreated TamR and wild-type MCF-7 cells treated with 20μM zinc (Taylor et al. 2008). TamR breast cancer cells have increased intracellular zinc, compared with their hormone-responsive (wild type) counterparts; accompanying this is an increased expression of ZIP7, a member of the LIV-1 family of ZIP zinc transporters, which is capable of increasing intracellular zinc concentration (Taylor et al. 2008).

3.8 The role of ZIP6 in breast cancer

ZIP6 transporter (SLC39A6), a plasma membrane bound member of the LIV-1 subfamily, was the first ZIP transporter to be linked to breast cancer. ZIP6, also called LIV-1 is oestrogen-regulated and is present in increased amounts in oestrogen-receptor positive breast cancers with an ability to spread to the lymph nodes (Manning et al. 1993). It is a reliable marker of oestrogen receptor positive cancers. This association has been substantiated by larger scale analysis of breast cancer specimens (Schneider et al. 2006). ZIP6 was shown to be the downstream target of the transcription factor STAT3 (signal transducer and activator of transcription 3) in zebrafish embryos (Yamashita et al. 2004). It was proven that STAT3 has a role in the development of cancer (Dauer et al. 2005). STAT3 does not alter mammary tumour initiation but greatly affects metastatic progression (Ranger et al. 2009). Once STAT3 has been activated, ZIP6, the

downstream target of STAT3, induces influx of zinc into the cells from its position on the plasma membrane (Taylor et al. 2004).

3.9 The role of STAT3

Signal transducers and activators of transcription (STATs) control cellular growth, differentiation, development and survival (Bowman et al. 2000). Persistent signalling of specific STATs directly contributes to oncogenesis through up-regulation of genes encoding apoptosis inhibitors and cell cycle regulators (Buettner et al. 2002). Constitutive activation of STAT3 was detected in breast carcinoma cell lines but not in normal breast epithelial cells (Garcia et al. 2001). Inhibition of this constitutive activation in tumour cell lines has been associated with growth suppression and induction of cell death. By contrast, tumour cells lacking STAT activation or normal cells are more tolerant to the small molecular inhibitors used in these experiments (Garcia et al. 2001, Catlett-Falcone et al. 1999). STAT3 exists at least in four isoforms (α, β, γ, δ). All STAT3 isoforms are potential targets for tyrosine phosphorylation of residue 705 (Hevehan et al. 2002). STAT3α (92 kDa) is a full-length isoform that is expressed in most cells (Kato et al. 2004). Only this form is subject to serine phosphorylation on residue 727 (Hevehan et al. 2002). STAT3β (83 kDa) is an alternatively spliced RNA form (Kato et al. 2004), in which the 55 amino acids from the C-terminus of STAT3α are replaced by 7 amino acid residues specific to STAT3β (Schaefer et al. 1997) (*Figure 4*). STAT3 becomes activated at the plasma membrane by phosphorylation on tyrosine residue (Tyr705) in the C-terminus (Zhong et al. 1994). The activated STATs dimerize via their own SH2 domains (Shuai et al. 1994), translocate into the nucleus, and bind specific DNA elements (Wen et al. 1995, Zhang et al. 1995). In vivo studies provided convincing evidence of another phosphorylation site, a serine residue at position 727 in the C-terminus (Wen et al. 1995). The transcriptional activities of serine-phosphorylation of STAT3, however, remain questionable. Some reports show a correlation of STAT3 serine phosphorylation with increased DNA-binding (Wen et al. 1995). Serine phosphorylation appears to be required for the formation of stable STAT3 homodimers and DNA complexes (Zhang et al. 1995, Ng and Cantrell, 1997). Another group of results suggests that phosphorylation on a single serine in each protein is also required for maximal transcriptional activity (Wen et al.

1995). Tyrosine-phosphorylation of STAT3 is not enhanced by a phosphorylated Ser727 (Decker and Kovarik, 2000). Chung et al. (1997) reported that STAT3 phosphorylation on Ser727 negatively modulates its tyrosine phosphorylation. Tyrosine-phosphorylation of STAT3 has been widely researched whereas serine-phosphorylation has been largely ignored. In this study however, the aim was to investigate the known association of ZIP6 and STAT3 in relation to mitosis and with regard to phosphorylation on both Tyr705 and Ser727, at the protein level. Previous observations in our laboratory have also shown that pSTAT3(Ser727) is activated throughout mitosis. It is proposed that both ZIP6 and STAT3 are involved in mitosis and the study of their role in this mechanism may lead to the identification of potential targets which would be beneficial for the development of new pharmacological treatments.

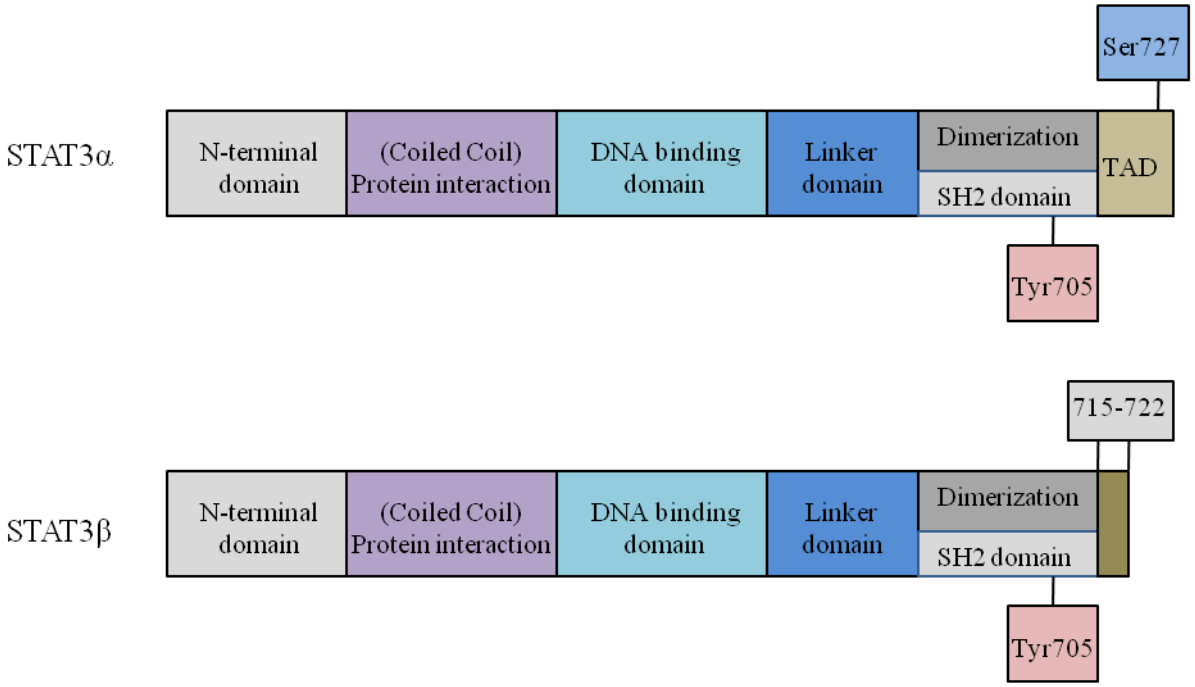

Figure 4: Schematic diagram showing structures of STAT3 proteins. STAT3α shows full-length STAT3 with both Ser727 and Tyr705 phosphorylation sites. Stat3β shows the C-terminally truncated protein, generated by alternative splicing of STAT3α, with the addition of 7 unique amino acids downstream of the deletion and loss of the transactivation domain (TAD). STAT3β cannot be serine-phosphorylated. It does not contain the Ser727 phosphorylation site (adapted according to Buettner et al. 2002).

4. Aims of the thesis

- To observe any association between ZIP6 and STAT3 during mitosis
- To investigate an association between N-terminal cleavage of ZIP6 and mitosis
- To observe the effect of zinc supplementation on cells in mitosis
- To observe the effects of the TPEN, STAT3 inhibitor and zinc on cells in mitosis

5. Materials and methods

5.1 Cell lines

5.1.1 MCF-7 cells

The Michigan Cancer Foundation is the institute in Detroit where the MCF-7 cell line was defined in 1973 by Herbert Soule (Soule et al. 1973). MCF-7 cells are useful for *in vitro* breast cancer studies because the cell line has retained several characteristics typical of mammary epithelium. One of these includes the ability of MCF-7 cells to process oestrogen, in the form of oestradiol, via oestrogen receptors in the cytoplasm. This makes the MCF-7 cell line an oestrogen receptor (ER) positive control cell line (MCF-7 Cells).

Wild type (tamoxifen responsive) MCF-7 breast cancer cells, obtained from AstraZeneca (Macclesfield, UK) were cultured in a monolayer in phenol-red-free RPMI 1640 medium[A] (Roswell Park Memorial Institute) supplemented with 5% (v/v) foetal calf serum (FCS) plus 4mM L-glutamine, 10IU/ml penicillin, 10µg/ml streptomycin and 2.5µg/ml fungizone.

5.1.2 Tamoxifen-resistant breast cancer cells (TamR)

To investigate the role of the zinc transporter, ZIP6, and STAT3 in mitosis, a tamoxifen-resistant breast cancer model (TamR) derived from the oestrogen receptor-positive human breast cancer cell line MCF-7 was used. This model was developed at the Tenovus Centre for Cancer Research to understand the mechanisms of anti-oestrogen resistance in breast cancer (Knowlden et al. 2003). Briefly, to develop tamoxifen resistance, the MCF-7 cell line was continuously exposed to 4-hydroxytamoxifen (10^{-7}M in ethanol) in phenol-red-free RPMI 1640 medium[A] with 5% charcoal-stripped steroid-depleted foetal calf serum (SFCS) and supplemented with penicillin-streptomycin (10IU/ml-10µg/ml), fungizone (2.5µg/ml) and L-glutamine (4mM). This treatment regimen was followed for six months until 70% confluence was achieved. At first, tamoxifen treatment reduced MCF-7 cell proliferation, but after two months, cell growth gradually increased, indicating the development of a cell line

resistant to the growth-inhibitory properties of 4-hydroxytamoxifen. TamR cell line was cultured for a further four months in medium containing 4-hydroxytamoxifen before characterization studies (Knowlden et al. 2003).

5.2 Culture conditions

Cells were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide (CO_2). The growth medium was replaced every four days. The cells were then passaged, using 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) to detach the cells. The trypsin was inactivated with an excess of medium, containing serum, and the cells were spun down at 1000rpm for 5 minutes in a Jouan C312 centrifuge. The supernatant was carefully removed, the cells resuspended in culture medium and seeded into Petri-dishes and incubated until 70% confluence was reached (approximately once a week). All tissue culture medium and constituents were purchased from Life Technologies Europe Ltd. (Paisley, Scotland) and tissue culture plasticware was obtained from Nunc (Roskilde, Denmark).

5.3 Nocodazole and zinc treatment

After achieving 70% confluence, TamR cells per 60mm dish were exposed to nocodazole, the inducer of mitotic arrest, at 100ng/ml for 20 hours. Additional treatments, in medium with 5%SFCS, for the last hour of this 20 hour nocodazole treatment were: 25 or 50μm *N,N,N'N'*-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) (the cell permeable zinc chelator) , 200μM STAT3 inhibitor, 20, 50 and 100μM zinc or 10μM pyrithione. As SFCS binds zinc, 100μM added zinc gives ~250nM free zinc, 50μM added zinc gives ~125nM free zinc (Cahyadi, 2011).

5.4 Cell harvest

TamR cells were grown in 60mm diameter dishes. Non-adherent cells in the medium were transferred to a universal container with ice-cold Dulbeccos' phosphate buffered saline (DPBS) and pelleted by centrifugation at 1000rpm for 5 minutes. Lysis of the adherent cells was performed by scraping them from the surface of the dish in 250µl ice-cold lysis buffer[B] containing phosphatase inhibitors[C] and a cocktail of protease inhibitors[D]. The partly lysed cells were then transferred to an Eppendorf tube. The pelletted non-adherent cells were also resuspended in the same lysis solution[E] and pooled with adherent cells. The samples continued to lyse on ice for 1 hour before centrifugation, for 15 minutes, at 4°C. The resulting supernatant was transferred to a new Eppendorf tube and stored at -20°C until required.

5.5 Protein assay

To determine the protein concentration in the samples a protein assay based on the Lowry Assay, utilizing Coomassie Brilliant Blue G-250 as the detection reagent, was carried out. This approach was essential to ensure an equal loading of samples into the wells and achieve comparable levels of sample proteins. The sample lysis buffer contained the detergent Triton X-100, therefore detergent compatible (DC) protein assay kit (Bio- Rad Laboratories Ltd., Hemel Hempstead, UK) was used. A standard curve was created using bovine serum albumin (BSA) prepared at concentrations of 0, 0.25, 0.5, 0.75, 1.0 and 1.45mg/ml. The samples were diluted 1:10 with sample lysis buffer[B].

250µl Reagent A (Bio-Rad DC™ Protein Assay) (containing 20µl Reagent S per 1ml) and 2ml Reagent B were added to different BSA concentrations and samples, mixed and left for 5 minutes to enable formation of the coloured product. The absorbance at 750nm was then measured on a BioMate 3S spectrophotometer (Thermo Fisher Scientific, Madison, USA). Protein concentrations of the samples were calculated using the BSA standard curve. The volume of each lysate required for loading into a lane for SDS-PAGE was then calculated giving an equal amount of protein per lane (20µg/lane, loading 20µl/lane) therefore making the results comparable.

5.6 Sample preparation for sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Samples were diluted in Laemmli sample loading buffer[H] that contains sodium dodecyl sulphate (SDS) an anionic detergent, which is necessary to break down the secondary and tertiary structure of the proteins. This approach converts the proteins into linear highly negatively charged polypeptides, making the migration dependent only on the molecular weight. The buffer also contains the reducing agent dithiothreitol (DTT) which breaks disulfide bonds between amino acids, bromophenol-blue that gives the samples a visible blue colour to track migration and glycerol which gives the solution a higher density than the running buffer and thus makes the sample sink to the bottom of the well. To prepare the samples for electrophoresis, the required volume of cell lysate containing 200µg was mixed with an equal volume of 2x loading buffer. 1x loading buffer was added to make the volume up to 200µl giving a protein concentration equal to 1µg/µl. The samples were then heated to 100°C for 5 minutes to denature the proteins completely.

5.7 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Using SDS-PAGE, all proteins have an equal charge/mass ratio and therefore separate under electrophoresis according to their molecular weight and not charge. Glass plates provided with the apparatus *(Figure 5)* were cleaned thoroughly with 70% ethanol. Once the apparatus was assembled, a lower resolving gel[F] (12% acrylamide/bis-acrylamide) was prepared. The lower gel was carefully applied between the glass plates of the apparatus, leaving approximately 2cm for the stacking gel and overlaid with isopropanol to get an even surface. This was allowed to set for at least 20 minutes. The lower resolving gel separates the proteins according to their size. The layer of isopropanol was poured off and the rest was carefully removed using filter paper. The upper stacking gel[G] (4%) was then added and a plastic comb, which forms the wells was immediately inserted. The stacking gel was allowed to set for 30 minutes. This gel[G] accumulates the proteins so that the bands become sharp. Additionally both gel

sections contain sodium dodecyl sulphate (SDS), ammonium persulphate (APS) and N,N,N´,N´-tetramethylethylenediamine (TEMED). TEMED initiates the gel polymerization and APS catalyzes the polymerization and should therefore be added at the end. Once the gel had set, the comb was removed and the electrophoresis apparatus was assembled. For running the gels a Biorad Mini-Protean III apparatus (Bio- Rad, Herefordshire, UK) was used. The gels were submerged in running buffer[I] and the wells were loaded with 20µl of each sample (equal to 20µg of protein) and 5µl of Precision Plus protein standards marker (Bio-Rad, Herefordshire, UK). The marker contains ten recombinant proteins that offer exceptional precision and consistency in their migration patterns and is designed to provide the most accurate protein reference bands for molecular weight estimation. The gels were run at 150 V constant until the tracker dye was approximately 1cm from the bottom of the gel (time differs according to the voltage and the percentage of the gel used).

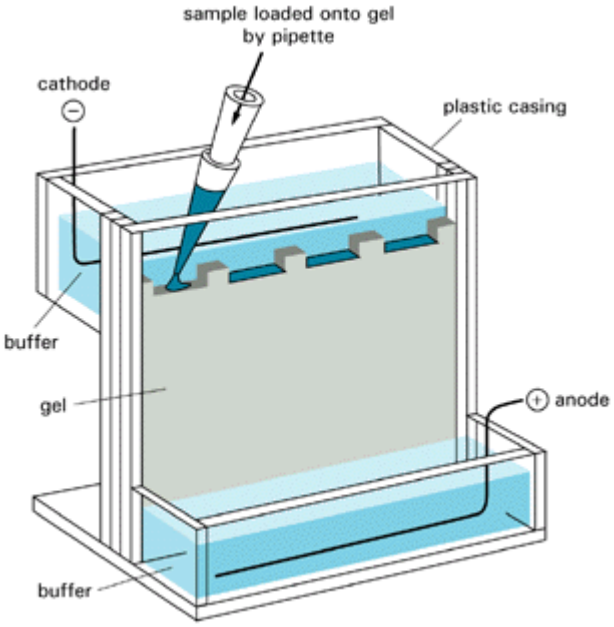

Figure 5: An apparatus used for SDS PAGE (adopted from the Jena Centre for Bioinformatics)

5.8 Western blotting

This method is a powerful and commonly used tool to identify a specific protein in a complex mixture. It can produce qualitative and semi-quantitative data about the proteins of interest (Thermo Scientific Pierce Protein Biology products).

The proteins, separated by SDS-PAGE, were electro-blotted onto a 0.45µM nitrocellulose transfer membrane (Schleicher and Schuell, Inc, Dassel, Germany) by Western blotting using a Bio-Rad "Mini Trans-Blot Cell" (Bio-Rad, Herefordshire, UK). Fibre sponges, filter papers and nitrocellulose membrane with polyacrylamide gel in the middle were sandwiched between a plastic cassette, as in the diagram (*Figure 6).* A pipette was drawn across the surface of the assembled sandwich with gentle pressure to ease out any air bubbles that formed between the layers as they can disrupt the transfer of proteins from gel to membrane. The plastic housing was then placed into the transfer apparatus with the gel nearest the negative electrode (cathode) and the membrane positioned nearest the positive electrode (anode). An ice block was inserted to prevent over-heating, the transfer tank filled with transfer buffer[J] and run at 100V constant for 60 minutes. The membranes were removed from the cassette, washed with distilled water and briefly stained with Ponceau S[K]. Ponceau S is a red diazo reversible dye that binds to all proteins and is used to visually confirm efficient transfer and even loading of protein. A photocopy of the Ponceau S staining was taken. Blots were rinsed with 1x Tris-buffered saline containing 0.05% Tween-20 (TBST)[L] to remove all Ponceau S.

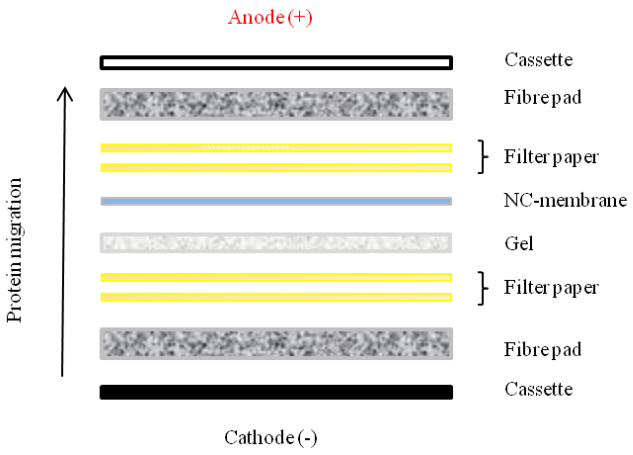

Figure 6: Diagram showing the arrangement of fibre pads, filter paper, nitro-cellulose membrane and gel in the transfer cassette.

5.8.1 Immuno-detection

In order to avoid non-specific signals and to reduce background as a result of cross-reactivity of the primary and the secondary antibody with other proteins or non-specific interactions between the unreacted sites on the membrane, it was necessary to block the membrane by incubating in 5% non-fat milk powder dissolved in TBST[L] (w/v) at room temperature (RT) for 30 minutes. Membranes were again washed in TBST (2x 5 minutes) before incubating overnight at 4°C with the primary antibody of interest. All primary antibodies were diluted in TBST with 5% Western Blocking Reagent (Roche Diagnostics GmbH, Mannheim, Germany) and 0.02M sodium azide to preserve the antibody. The next day, the blots were washed in TBST (3x 5 minutes) at RT, on a rocking platform then incubated with secondary antibody (anti-mouse or anti-rabbit horseradish peroxidase-conjugated IgG) diluted 1 in 10000 with 1% Marvel/TBS-Tween (w/v) for 1 hour. After this, the blots were again washed on a rocking platform in TBST (5x 5 minutes). The antibodies used in this study are summarized in *Table 2*.

5.8.2 Chemiluminescent-detection

Different detection kits were used according to the strength of signal. Three chemiluminescent detection kits were available - SuperSignal West Pico Chemiluminescent Substrate – for larger (picogram) amounts of proteins, West Dura Extended Duration Chemiluminescent Substrate - was mostly used as it detects protein amounts in the mid-femtogram to low picogram range and its signal remained stable for approximately 24 hours. For lower protein amounts (down to 1 femtogram) West Femto Maximum Sensitivity Chemiluminescent Substrate was used. These kits consist of two reagents, luminol and hydrogen peroxide, which were mixed together in equal amounts and applied to the membrane between a plastic folder. This allowed a chemical reaction between the HRP-labelled secondary antibodies and the luminol/hydrogen peroxide substrate solution and the formation of the excited product 3-aminopthalate. As it decays to the ground state, 3-aminopthtalate emits light at 425nm, which was detected when blots were exposed to X-ray film (MXB Kodak film) in the dark room in a light excluding cassette. After a certain time (exposure time was found experimentally) the film was developed by X-Ograph Compact X2 developer. For quantification, the signals

produced on film were scanned using an imaging densitometer and analysed using AlphaDigiDoc software (The AlphaEaseFC Stand Alonefor Windows 2000/XP). Protein levels estimated by densitometry were corrected relative to the loading control glyceraldehyde-3-phosphate (GAPDH).

5.8.3 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

GAPDH is expressed at high and usually constant levels in almost all tissues and cell lines, making it useful as a loading control in immuno-blots.

The nitrocellulose membrane was incubated in GAPDH antibody, 1 in 50000 dilution, at RT for 30 minutes. The membranes were washed in TBST (3x 5 minutes) and then incubated with HRP-conjugated anti-mouse antibody diluted 1 in 10000 with 1% non-fat milk powder dissolved in TBST (w/v). The blots were subsequently washed with TBST (5x 5 minutes) and the signal detected using the Pico chemiluminescent kit.

After quantification of bands by densitometry GAPDH was used to correct the protein signal of interest by showing it as the ratio of the GAPDH signal.

5.8.4 Calculation of the molecular weight of proteins

The distance travelled by the bromophenol blue dye and the proteins (molecular weight markers and proteins of interest) was measured from the top of the resolving gel. The Rf (relative mobility) values were calculated using the formula: Rf = distance travelled by the protein/ the distance travelled by the dye. The Rf values of the molecular weight markers were plotted against their molecular weights. This graph was then used to determine the molecular weights of the proteins detected with LIV-1 M6, LIV-1 SC and LIV-1 Y3 antibodies.

5.9 Fluorescent microscopy imaging

Working in the sterile hood TamR cells cultured on 0.13mm thick glass coverslips were fixed in 3.7% (v/v) formaldehyde in phosphate buffered saline (PBS) for 15 minutes. After washing in PBS, the coverslips were incubated in permeabilisation buffer[N] for 15 minutes to allow access of the antibody through the cell membrane. Coverslips were then blocked with 10% normal goat serum in PBS (v/v) for 15 minutes and incubated with mouse and rabbit primary antibodies[M] for 1 hour at RT. Before the secondary antibodies were added the coverslips were washed three times with permeabilising buffer. The secondary antibodies, either Alexa Fluor 488 goat anti-rabbit IgG (1 in 1000) (green-fluorescent) for the rabbit primary antibody, Alexa Fluor 594 goat anti-mouse IgG (1 in 2000) (red-fluorescent) for the mouse primary antibody were added for 30 minutes at RT. The coverslips were kept dark under foil to preserve the fluorescence. Slides were labelled to identify the coverslips. One drop of vectorshield mounting medium with 4,6-diamino-2-phenylindole (DAPI) (Vector Laboratories, Peterborough, UK) which was used to counterstain the nucleus was placed on each slide. The coverslips were then drained and mounted on the slide with the Vectorshield and the edges of the coverslips were sealed with a generous coating of clear nail varnish. The slides were viewed on a Leica RPE automatic microscope using a x63 oil immersion lens and processed with one round of deconvolution. PaintShop Pro was used to separate the colours before presenting in Powerpoint.

5.10 Statistics

The means of separate experiments were analysed using paired t-tests. If $p<0.05$, statistical significance was assumed. Where indicated, values are presented ± SE.

[A] Phenol red-free RPMI 1640 medium

Phenol red-free RPMI	100ml
Glutamine (200mM)	2ml
Charcoal-stripped foetal calf serum (ie steroid-depleted) (SFCS)	5ml
Penicillin/streptomycin (100IU/ 100µg/ml)	1ml
Fungizone (amphotericin B 250µg/ml…1ml in 100ml of RPMI)	1ml

[B] Lysis buffer	**100ml**
50mM TRIS base	0.61g
5mM ethylene glycol tetraacetic acid (EGTA)	0.19g
150mM NaCl	0.87g
1% Triton X-100	1ml

pH 7.5, stored at 4°C, add dH_2O to 100 ml

[C] Phosphatase inhibitors

2mM $NaVO_4$ (100 mM stock in water)

250mM NaF (2.5 M stock in water)

[D] Protease inhibitor cocktail

0.1mM phenylmethylsulfonyl fluoride (PMSF) (100mM stock in isopropanol)

20µM phenylarsine (20 mM stock in chloroform)

10mM sodium molybdate (1 M stock in water)

4µg/ml leupeptin (5mg/ml stock in water) stored at -20˚C

10µg/ml aprotinin (2mg/ml strock in water) stored at -20˚C

[E] Complete lysis solution	**10ml**	**Final conc**	
$NaVO_4$ (100mM stock in water)	200µl	2mM	
NaF (2.5M stock in water)	100µl	50mM	
PMSF (100mM stock in isopropanol)	10µl	1mM	add just before use
Phenylarsine (20mM stock in chloroform)	10µl	20µM	
Sodium molybdate (1M stock in water)	100µl	10mM	
Leupeptin (5mg/ml stock in water)	20µl	10µg/ml	
Aprotinin (2mg/ml strock in water)	50µl	8µg/ml	
Lysis buffer	9.51ml		

[F] Resolving (lower) gel (12%) – enough for 2 x 1.5mm gels

Distilled H_2O (dH_2O)	6.6ml
Lower buffer (1.5M TRIS pH 8.8)	5.0ml
Acrylamide/bis acrylamide 30%	8.0ml
Sodium dodecyl sulphate (SDS) 10%	200µl
Ammonium persulphate (APS) 10%	200µl
N,N,N′,N′-tetramethylethylenediamine (TEMED)	15µl

Mix gently and add TEMED just before use

[G] Stacking (upper) gel (4%) – enough for 2 x 1.5mm gels

Distilled H_2O (dH_2O)	6.1ml
Upper buffer (0.5 M TRIS pH 6.8)	2.5ml
Acrylamide /bis acrylamide 30%	1.3ml
Sodium dodecyl sulphate (SDS) 10%	100µl
Ammonium persulphate (APS) 10%	50µl
N,N,N´,N´-tetramethylethylenediamine (TEMED)	15µl

Mix gently and add TEMED just before use

[H] 2x Laemmli sample loading buffer	**10ml**
Sodium dodecyl sulphate (SDS) 10%	4ml
Glycerol	2ml
1M TRIS (pH 6.8)	1.2ml
Distilled H_2O	2.8ml
Bromphenol blue	0.01%

Prepare buffer without 0.1M DTT: add 16mg to 1ml 2x loading buffer just before use

[I] 10x running buffer	**1 l**
0.25M TRIS base	30g
1.92M glycine	144g
0.1% sodium dodecyl sulphate (SDS)	10g
Distilled H_2O	up to 1 l

pH 8.3 dilute 10x before use to give 1x concentrated running buffer

[J] Transfer buffer	**1 l**
0.25M TRIS base	3.03g
1.92M glycine	14.4g
20% methanol	200ml
Distilled H_2O	800ml

[K] Ponceau S

0.1% Ponceau S in 5% acetic acid (w/v)

[L] 10x TBS Tween	**1 l**
10mM TRIS	12.1g
100mM NaCl	58g
0.1% Tween 20	5ml
Distilled H_2O	up to 1 l

Adjust pH to 7.6 with 5M HCl (approx. 15ml)

Dilute 10x before use to give a 1x concentrated solution of TBS Tween

[M] Antibody diluent	**10ml**
1x TBS Tween	9.4ml
Western blocking reagent (WBR)	500µl
Sodium azide (1M NaN_3)	100µl

[N] Permeabilisation buffer	**100ml**
Bovine serum albumin (BSA)	1g
Saponin	0.4g
Phosphate buffered saline (PBS)	up to 100ml

*Table 2:*Antibodies

Antibody	Animal	Dilution	Supplier
pSTAT3(Ser727) (6E4) #9136S monoclonal	Mouse IgM	1 in 1000	Cell Signaling Technology
pSTAT3 (Ser727) #9134S polyclonal	Rabbit	1 in 1000	Cell Signaling Technology
p-Histone H3 (Ser10) (D2C8) XP® #3377S monoclonal	Rabbit IgG	1 in 2000	Cell Signaling Technology
pSTAT3(Tyr 705) #E0511 polyclonal	Rabbit IgG	1 in 1000	Santa Cruz Biotechnology
STAT3 (H-190) sc-7179 #A2704 polyclonal	Rabbit	1 in 2000	Santa Cruz Biotechnology
LIV-1 M6	Mouse	1 in 1000	Biogenes, Germany
LIV-1 Y3	Mouse	1 in 1000	Biogenes, Germany
LIV-1 SC (E-20) #J2209 polyclonal	Rabbit IgG	1 in 1000	Santa Cruz Biotechnology
GAPDH (6C5) sc-32233 monoclonal	Mouse IgG1	1 in 50 000	Santa Cruz Biotechnology
PP2A-β56-γ (H-40) #D2508 polyclonal	Rabbit IgG	1 in 1000	Santa Cruz Biotechnology
V5 R960-25	Mouse IgG2a	1 in 2000	Invitrogen
Horseradish peroxidase-labelled donkey #7074	Anti-rabbit IgG	1 in 10000	Cell Signaling Technology
Horseradish peroxidase-labelled sheep NXA931	Anti-mouse IgG	1 in 10000	GE Healthcare Limited
Alexa Fluor 488 A-11008	Goat anti-rabbit IgG	1 in 1000	Invitrogen
Alexa Fluor 594 A-11005	Goat anti-mouse IgG	1 in 2000	Invitrogen

6. Results

pSTAT3(Ser727), p-Histone H3(Ser10) and zinc transporter ZIP6 are present in mitotic cells

In order to examine STAT3 and ZIP6 expression and their potential role during mitosis, TamR cells were treated with/without nocodazole for 20 hours and examined by fluorescent microscopy. These results showed that 40% of nocodazole treated cells were in mitosis compared to 15% of untreated, control cells (Taylor, unpublished) *(Figure 7).*

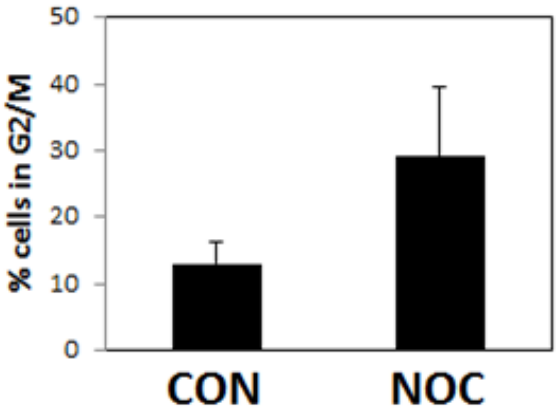

Figure 7: Mitotic content of nocodazole treated cells

Cells treated for 20 hours with/without nocodazole (noc/con) were analysed for the percentage of cells in G2/M phase, by fluorescent microscopy. Nocodazole treated cells showed an increase in mitotic percentage (Taylor, unpublished).

Subsequently, to confirm these observations, the effect of nocodazole on STAT3 and ZIP6 by Western blotting was examined. Since mitotic cells are only loosely attached to the culture dish, it is suggested that levels of STAT3 and ZIP6 proteins may be higher in non-adherent cells. Due to the low numbers of non-adherent cells, these cells were pooled with the adherent cells of the respective dish (pool) and compared to adherent cells alone (adherent). Cells were treated with nocodazole, which inhibits tubulin polymerisation and is widely used in cancer research to arrest and synchronise cells in mitosis. It was observed that p-Histone H3(Ser10) was significantly increased in nocodazole treated cells ($p<0,001$), confirming that a higher number of these cells were in mitosis in both pooled and adherent cell populations. pSTAT3(Ser727) was significantly increased in nocodazole treated cells but higher activation was seen in the pooled cells compared to the adherent ($p<0,05$). In contrast, pSTAT3(Tyr705) was reduced in nocodazole treated cells. Fluorescent microscopy results showed that 40% of cells were in mitosis compared to 15% control (Taylor, unpublished). That is the reason why we see both pSTAT3(Ser727) and pSTAT3(Tyr705) on blots (*Figure 8*). The increase in pSTAT3(Ser727) and decrease in pSTAT3(Tyr705) activation upon nocodazole treatment indicates that these phosphorylation states are reciprocally activated. In the nocodazole treated pooled population there was a higher level of ZIP6 than in the untreated. A blot probed for LIV-1 M6 antibody showed a 35 kDa band, which represented the N-terminal cleaved domain essential for relocation of ZIP6 to the plasma membrane. Additionally a strong 68 kDa band was detected with LIV-1 SC and LIV-1 Y3 antibodies which represented the remainder of LIV-1 after N-terminal cleavage, suggesting relocation to the plasma membrane. A 50 kDa band was also detected on the blot probed with the LIV-1 SC antibody (*Figure 8*), which would be consistent with the remainder of the N-terminal sequence of ZIP6 after cleavage 2 (*Figure 9*). Interestingly, this band was mainly observed in mitotic conditions.

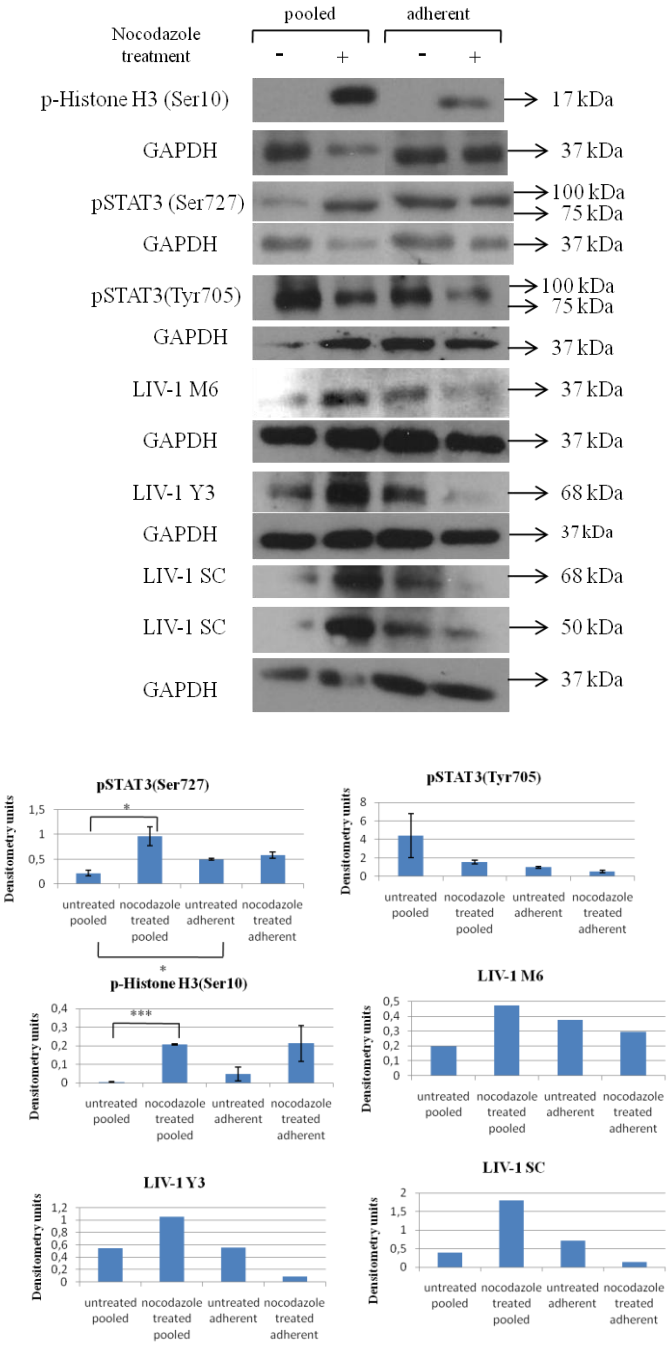

Figure 8: ZIP6 processing in mitosis

TamR cells, untreated or nocodazole-treated (100ng/ml) for 20 hours, were investigated by Western blotting. Adherent cells or a population of pooled adherent and non-adherent cells were assessed. Protein levels were assessed by densitometry and normalised to GAPDH. Representative blots of p-Histone H3(Ser10), pSTAT3(Ser727), pSTAT3(Tyr705) of three replicates ± SE are shown. A paired t-test was carried out (* $p<0,05$, *** $p<0,001$). And blots probed for in house ZIP6 antibodies LIV-1 M6, LIV-1 Y3 and LIV-1 SC are shown.

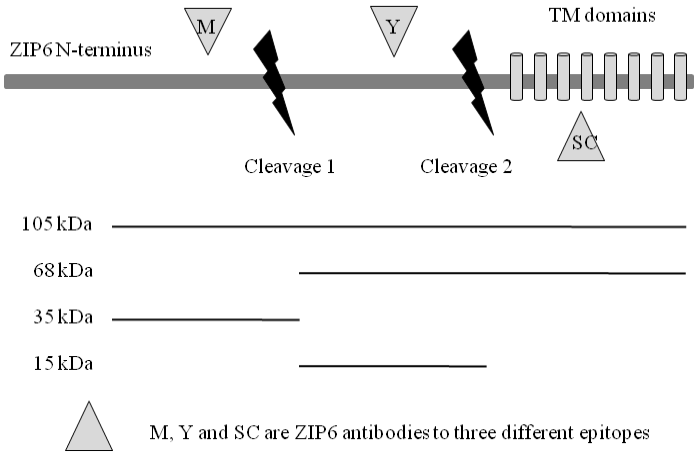

Figure 9: Schematic picture showing the location of the three ZIP6 antibody epitopes

The size of the bands were calculated from Rf (relative mobility) values. Cleavage 1 occurs in the endoplasmic reticulum to enable relocation to the plasma membrane. Cleavage 2 occurs in mitosis, releasing the remainder of the N-terminal sequence of ZIP6.

Rf values were calculated from western blots and used to work out the actual sizes of the bands (*Figure 10*). The M6 antibody detected the full length LIV-1 and a 35 kDa N-terminal fragment. LIV-1 Y3 also detected the full length protein and a 68 kDa cleavage product and like the other two antibodies, LIV-1 SC detected the full length protein as well as a 68 kDa product after N-terminal cleavage (*Figure 8*). In addition LIV-1 SC also recognised a 50 kDa band in mitosis which represented the remainder of the protein after N-terminal cleavage 2 (*Figure 9*).

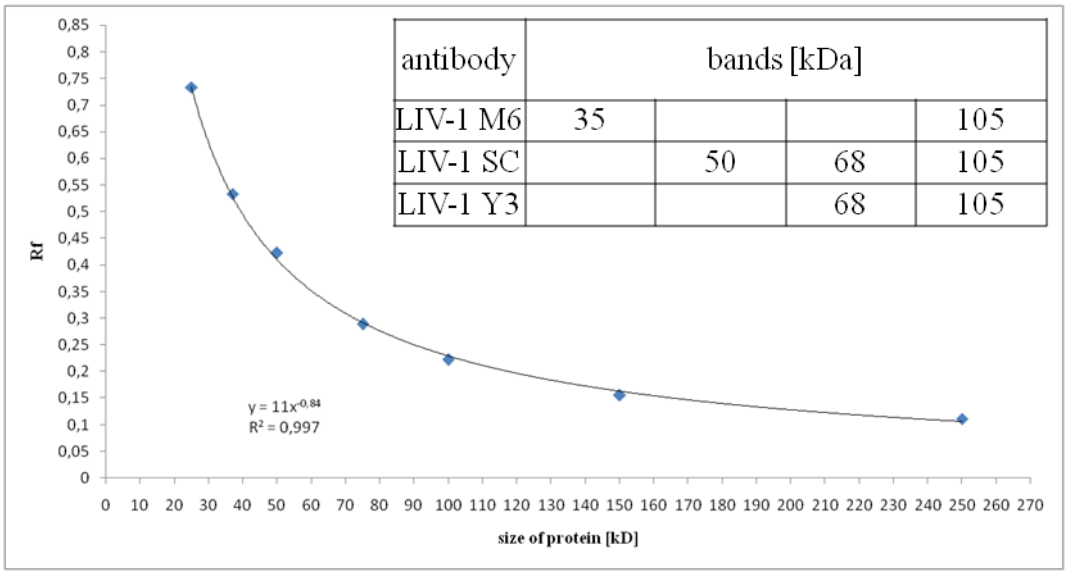

Figure 10: Calculation protein sizes according to formula of graph

The sizes of the bands were calculated from the Rf values and were read from the graph shown.

Effect of zinc and STAT3 on mitosis

The effect of zinc addition and chelation on TamR cells in nocodazole-induced mitotic arrest was examined (*Figure 11*). Cells were treated for 19 hours with nocodazole followed by 1 hour with nocodazole ± TPEN (specific zinc chelator), STAT3 inhibitor or zinc and pyrithione (P). Both the STAT3 inhibitor and suppression of zinc appeared to have no effect on the pSTAT3(Ser727) and p-Histone H3(Ser10) phosphorylation status, suggesting that zinc and STAT3 are required before the cells reach mitosis because these cells were already blocked in mitosis.

However, treatment of cells with 50μM and 100μM zinc in the last hour of their nocodazole treatment significantly reduced the expression of p-Histone H3(Ser10) ($p<0.05$), suggesting that the cells were no longer in mitosis. This conclusion would also fit with the fact that the pSTAT3(Ser727) was significantly reduced ($p<0,05$) (*Figure 11*). Interestingly, however, detection of total STAT3 also revealed different sized bands, the largest of which showed similar expression to that of full length STAT3 (*Figure 4*) which decreased considerably in zinc supplemented cells. The smaller band, consistent with a C-terminal cleaved STAT3 (*Figure 4*) was evenly expressed throughout the samples. It is also of interest that, when STAT3 is cleaved at the C-terminal end this will result in the removal of Ser727 and prevent its phosphorylation. These results therefore suggest that zinc treatment may have accelerated the transit through mitosis and caused cleavage of STAT3 as a means to remove the serine 727 phosphorylated form of STAT3, thus ending mitosis.

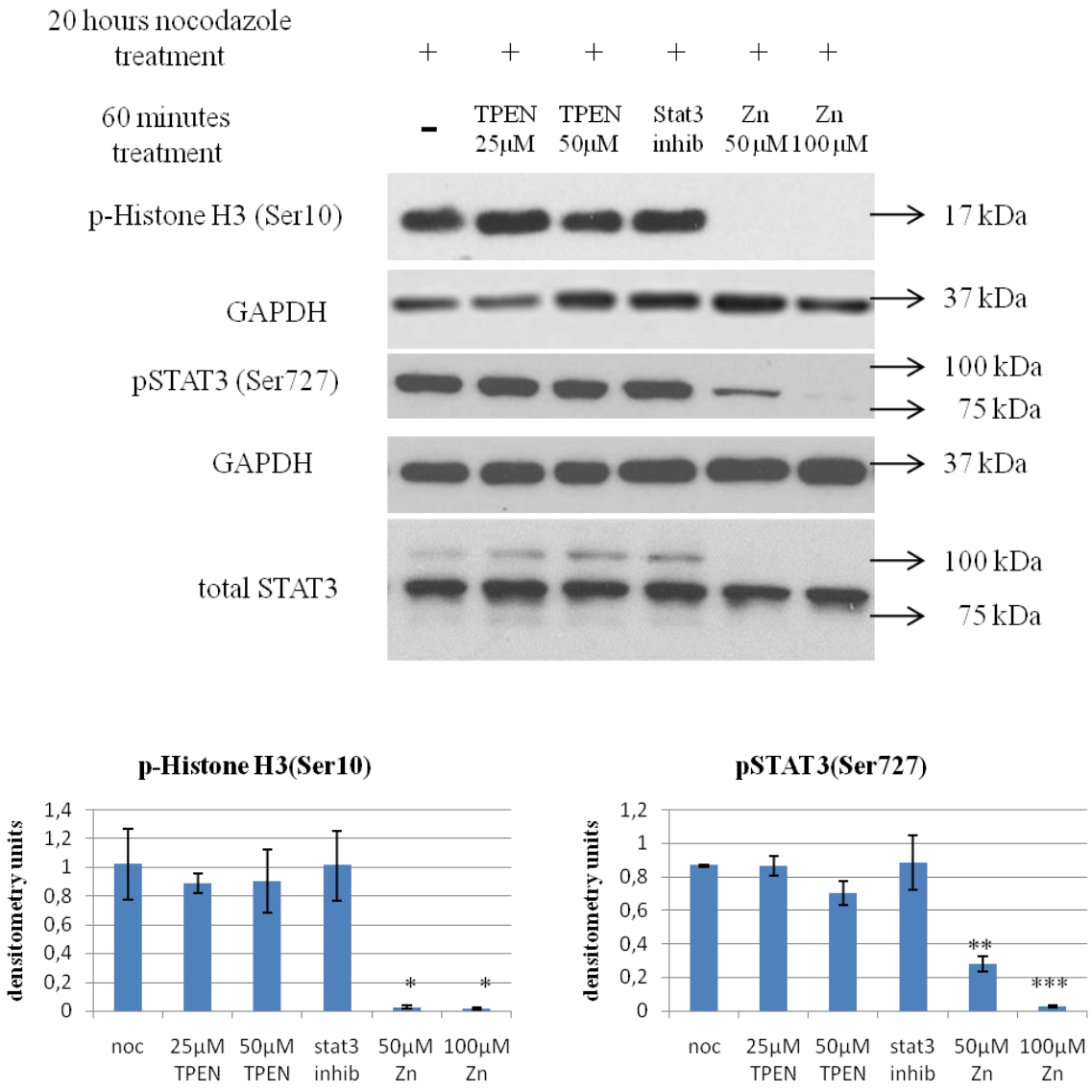

Figure 11: Zinc addition speeds up mitosis

Pooled adherent and non-adherent cells, from TamR cultures, were treated with nocodazole (noc) for 19 hours followed by 1 hour with nocodazole ± TPEN, STAT3 inhibitor, zinc and pyrithione (P) in 5%SFCS medium. Protein levels were assessed by densitometry, normalised to GAPDH and the blot shown is representative of three replicates ± SE. A paired t-test was carried out (* $p<0,05$, ** $p<0,01$, *** $p<0,001$).

Effect of zinc treatment on nocodazole treated cells

We used p-Histone H3(Ser10) as an indicator of mitotic cells and PP2A-β56γ as an indicator of non-mitotic cells (*Figure 12*). Addition of 20μM zinc to TamR cells treated with nocodazole, to induce mitosis, increased the expression of pSTAT3(Ser727) and decreased the expression of pSTAT3(Tyr705). Zinc is known to alter the tertiary structure of STAT3 and prevents it becoming tyrosine-phosphorylated (Kitabayashi et al. 2010). This may be responsible for the reciprocal activation of pSTAT3(Tyr705) and pSTAT3(Ser727) in mitosis. These results demonstrated that zinc may be involved in this mechanism. However, addition of zinc at 100μM reduced the expression of pSTAT3(Ser727) and p-Histone H3(Ser10) which agrees with the cells moving through and out of mitosis (*Figure 12).*

A blot probed for LIV-1 M6 antibody showed 35 kDa band, which represents the N-terminal cleaved domain essential for relocation of ZIP6 to the plasma membrane. These bands mirrored the profile seen for p-Histone H3 (Ser10), which proves that cells were in mitosis. We observed a 68 kDa band for LIV-1 SC antibody which represents the remainder of the protein after N-terminal cleavage, suggesting relocation to the plasma membrane. The expression of this protein shared the same profile as p-Histone H3(Ser10) indicating mitosis (*Figure 12*).

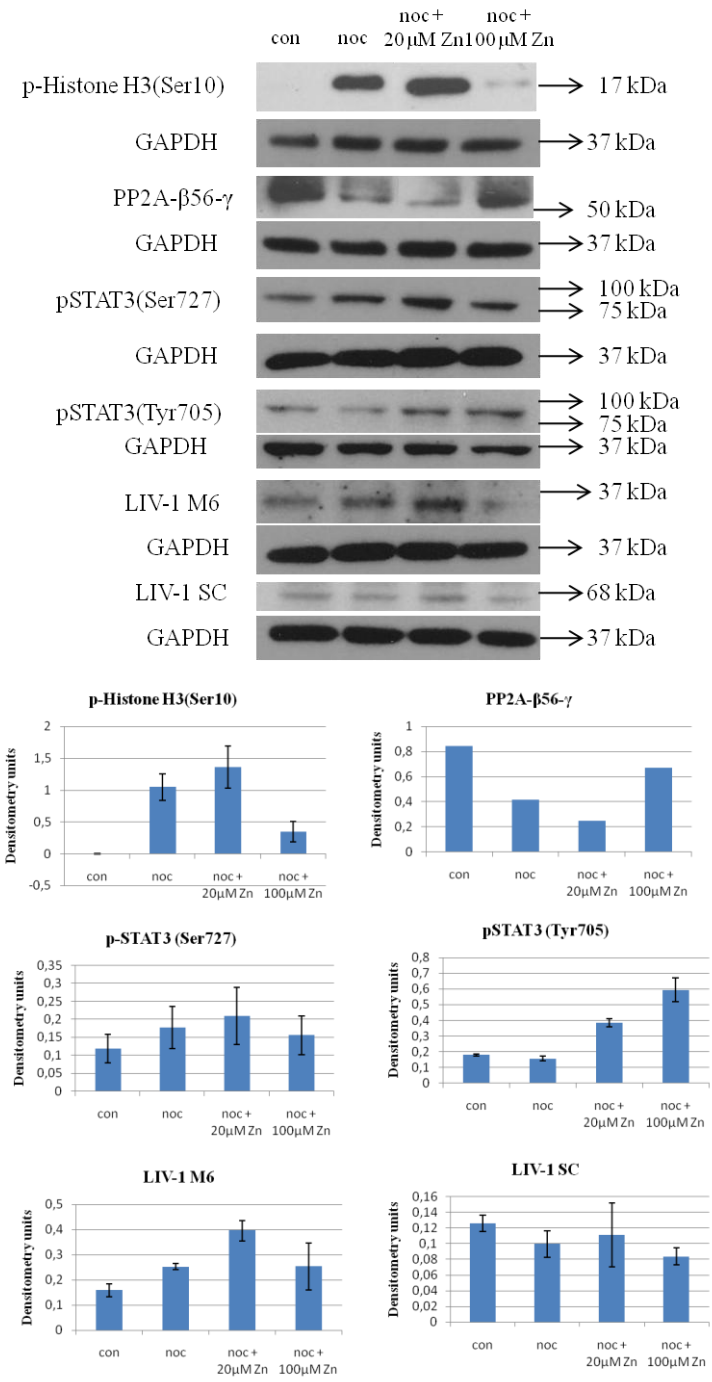

Figure 12: The effect of zinc on mitosis

Pooled adherent and non-adherent cells, from TamR cultures, were treated for 20 hours with/without nocodazole (noc/con) or with nocodazole including treatment with 20µM zinc/100µM zinc in serum-free medium for the last 20 minutes. Protein levels assessed by densitometry were normalised to GAPDH. Shown is a blot representative of p-Histone H3(Ser10), pSTAT3(Ser727), pSTAT3(Tyr705), LIV-1 M6, LIV-1 SC of two replicates ± SE and PP2A-β56γ.

Zinc treatment causes pSTAT3(Tyr705) to be converted to pSTAT3(Ser727)

Addition of 20μM zinc with pyrithione in serum-free medium for just 20 minutes significantly increased the phosphorylation of pSTAT3(Ser727) ($p<0.05$) and significantly decreased that of pSTAT3(Tyr705) ($p<0.05$) (*Figure 13)*. Without zinc treatment STAT3 was present as pSTAT3(Tyr705) with only a little amount of pSTAT3(Ser727). However, after 20 minutes of zinc treatment, this was reversed with a significant increase in pSTAT3(Ser727) and decrease in pSTAT3(Tyr705). These results confirm the importance of zinc and its involvement with STAT3 phosphorylation for mitotic progression.

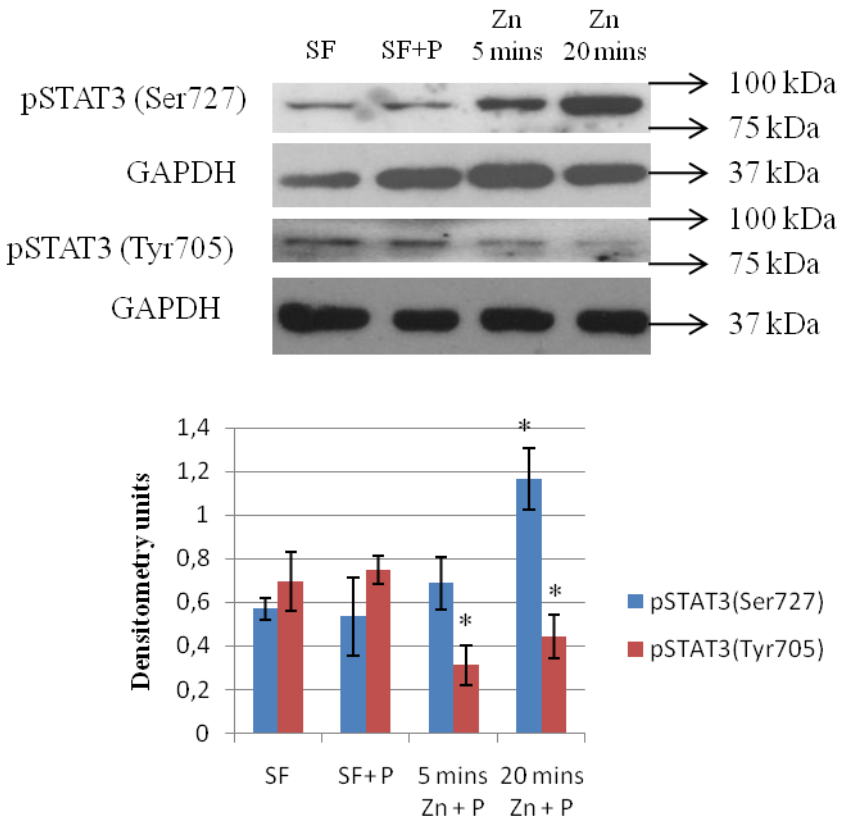

Figure 13: Zinc causes pSTAT3(Tyr705) to be converted to pSTAT3(Ser727)

TamR cells were treated with serum-free medium (SF) or serum-free medium and pyrithione (SF+P) for 20 minutes. Other samples were supplemented with 20µM zinc for 5 and 20 minutes. Protein activation levels of pSTAT3(Ser727) and pSTAT3(Tyr705) were assessed by densitometry, corrected for protein loading as ratio of GAPDH and shown is a blot representative of three replicates ± SE. A paired t-test was carried out (* $p<0,05$).

Fluorescent imaging of ZIP6 processing in cells

ZIP6 is thought to be cleaved on the N-terminus and directed from the endoplasmic reticulum to the plasma membrane, where it has N- and C-terminus which are localised extracellularly.

Panel A shows the full length ZIP6 present in the endoplasmic reticulum which is consistent with no mitosis (interphase) as seen from the DAPI staining and negative for pSTAT3(Ser727).

Panel B shows a cell at the start of mitosis in prophase also negative for pSTAT3(Ser727) and yet ZIP6 appears on the plasma membrane, consistent with N-terminal cleavage. This suggests that ZIP6 causes the cell rounding for mitosis before STAT3 becomes phosphorylated on Serine 727.

Panel C shows a mitotic cell as indicated by DAPI, that is positive for pSTAT3(Ser727), confirming the role of pSTAT3(Ser727) in mitosis. Unfortunately this cell was not probed for ZIP6 which would have been done with more time available (*Figure 14*).

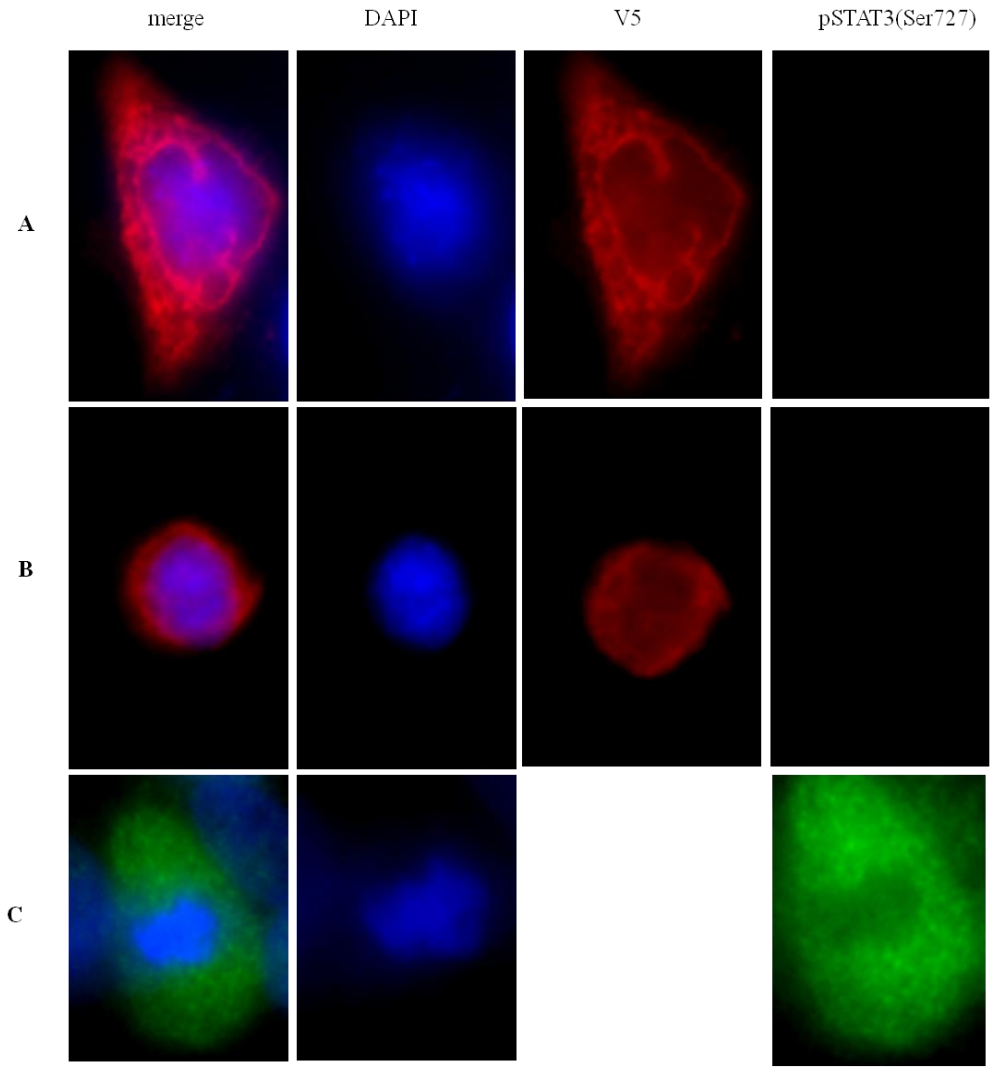

Figure 14: Fluorescent microscopy of recombinant ZIP6

ZIP6 is detected with V5 mouse primary antibody and Alexa Fluor 594 goat anti-mouse IgG (1 in 2000) (red-fluorescent) secondary antibody. Cells were also stained with pSTAT3(Ser727) rabbit primary antibody and Alexa Fluor 488 goat anti-rabbit IgG (1 in 1000) (green-fluorescent) secondary antibody. The nucleus was counterstained with DAPI. Panel A shows a non-mitotic cell, negative for pSTAT3(Ser727), when ZIP6 is localised in endoplasmic reticulum. Panel B shows a cell at the start of mitosis in prophase, it is rounded with ZIP6 located on the plasma membrane. The cell is negative for pSTAT3(Ser727), suggesting that ZIP6 is activated before STAT3 gets involved. Panel C shows a mitotic cell, positive for pSTAT3(Ser727).

7. Discussion

Even though the evidence for the phosphorylation of STAT3 on Ser727 emerged over 18 years ago (Wen et al. 1995), the functional consequence of phosphorylation at this site and the relationship to Tyr705 phosphorylation have remained relatively unknown. Our unique observations show that pSTAT3(Ser727) is present in mitotic cells (*Figure 11*). This finding is supported by a previous study on nocodazole-treated HeLa cells, which revealed that STAT3(Ser727) phosphorylation was enhanced during mitosis (Shi et al. 2006). There is also further evidence to support the association of pSTAT3(Ser727) with mitosis in breast cancer and normal mouse intestine (Taylor, unpublished). Mitotic cells are localised within intestinal crypts and it was found that the mitotic cells that were positive for p-Histone H3(Ser10) were also positive for pSTAT3(Ser727). This simultaneous immunostaining of p-Histone H3(Ser10) and pSTAT3(Ser727) was also observed in breast cancer tissue, providing further support for the presence of pSTAT3(Ser727) in mitotic cells. In contrast, these studies also showed the absence of pSTAT3(Tyr705) in mitosis. This is a potentially important discovery due to the extensive role of STAT3 in cancer. The reason why pSTAT3(Ser727) was overlooked in the scientific literature could be explained in a number of ways. Mitotic cells are loosely attached when grown in culture and may easily be lost from harvested cells. Furthermore, mitosis may only represent 1 hour in 24 hours of the normal cell cycle (Alberts et al. 2002) considerably reducing the amount of pSTAT3(Ser727) available compared to the pSTAT3(Tyr705).

In contrast to pSTAT3(Ser727), increased activation of STAT3 on Tyr705 was seen in untreated non-mitotic TamR cells *(Figure 8*), suggesting that pSTAT3(Tyr705) and pSTAT3(Ser727) are reciprocally regulated. These results support another study (Shi et al. 2006) where a similar correlation in phosphorylation state was exhibited in nocodazole-treated Hela cells. Chung et al. (1997) reported an increase in EGF-stimulated tyrosine-phosphorylation of STAT3 in a Ser727Ala mutant, compared to the STAT3 wild-type, suggesting that pSTAT3(Tyr705) is negatively regulated by pSTAT3(Ser727). Furthermore pSTAT3(Ser727) enhances dephosphorylation of pSTAT3(Tyr705) largely through the protein phosphatase TC45 in HepG2 cells (Wakahara et al. 2012).

It has been observed previously that zinc binds to pSTAT3(Tyr705) and changes its conformational shape (Kitabayashi et al. 2010) reducing the phosphorylation of STAT3(Tyr705). However, in this study we have extended this investigation further by observing that zinc treatment not only reduces pSTAT3(Tyr705) it actually increases pSTAT3(Ser727) (*Figure 13*). Therefore, it appears that zinc is involved in the increase of pSTAT3(Ser727) associated with mitosis by binding to STAT3 and changing its phosphorylation state.

Zinc has long been implicated in the cell cycle since Fujioka and Lieberman (1964) provided evidence of a zinc requirement for DNA synthesis. However, it is more likely that zinc is required for the passage of cells through the mid-G1 phase of the cell cycle since if it is unavailable, entry into S phase will be blocked. Zinc-dependence has also been shown in G2/M phase of a mammalian cell line. Cells arrested in G1/S phase, before release by addition of serum-containing medium, completed S, G2 and M phase. However, if the cells were released from cell cycle arrest in zinc-chelating diethylenetriaminepentaacetic acid (DTPA) medium, the cells appeared to complete the S phase but became trapped in G2 phase (Chesters and Petrie, 1999). The addition of zinc abolished the effect of the chelator and allowed cells to pass through G2, suggesting zinc is necessary at this stage in order for the cells to progress to mitosis. It also indicates a requirement for extracellular zinc, as DTPA is an extracellular zinc-chelating agent, whereas TPEN, the zinc specific chelator used in this study, is cell permeable. This requirement for extracellular zinc to progress through G2 phase indicates that plasma membrane located zinc transporters are needed to facilitate this.

Previously, ZIP6 (LIV-1) was shown to be the downstream target of the transcription factor STAT3 in zebrafish embryos (Yamashita et al. 2004). ZIP6 actually binds pSTAT3(Ser727) specifically in mitotic cells, suggesting participation in the serine-phosphorylation of STAT3 associated with mitosis (Taylor, unpublished). Cell cycle analysis of ZIP6 transfected MCF-7 cells showed an increase in the number of cells in G2/M phase compared to the non-transfected controls, suggesting that the presence of ZIP6 can increase the number of mitotic cells. These observations indicated an important role for ZIP6 in mitosis (Taylor, unpublished). In this study it is proposed that the influx of zinc causes a switch in phosphorylation state from pSTAT3(Tyr705) to

pSTAT3(Ser727) (*Figure 13*), facilitating binding of pSTAT3(Ser727) and ZIP6 in mitosis (Taylor, unpublished).

This raises the question as to how the ZIP6-mediated influx of zinc is regulated so that its function is only carried out at the onset of mitosis. ZIP6 must be localised on the plasma membrane, in order for zinc to be imported into the cell in this way. Using antibodies which target different residues along the N-terminus of ZIP6, it was discovered that this transporter resides in two cellular locations. Full-length ZIP6 was located on the endoplasmic reticulum while an N-terminal cleaved form was present on the plasma membrane (Taylor, unpublished). This led to the conclusion that ZIP6 is a pro-protein that is activated on relocation to the plasma membrane, therefore suggesting a mechanism by which zinc influx is limited to the onset of mitosis. The trigger for this mechanism however is not known. Furthermore, we recognised by our LIV-1 SC antibody an additional second N-terminal cleavage of ZIP-6 in a mitotic-related manner which produces a band of mass 50 kDa (*Figure 8*) and 15 kDa (Taylor, unpublished). This cleavage 2 corresponds with the ectodomain shedding previously observed for ZIP4 (Kambe and Andrews, 2009) and ZIP10 (Ehsani et al. 2012) and emphasises the relationship between prion proteins and ZIP transporters confirming that ZIP6 fills a basic cellular need, such as mitosis (Schmitt-Ulms et al. 2009).

These results are suggestive of the unknown mechanism for the role of zinc in mitosis. Therefore we next tried to examine whether altering the zinc level could affect the mitotic state of the cells. Both zinc chelation by TPEN and STAT3 inhibitor appeared to have no effect on the activation of pSTAT3(Ser727) after cells had been treated with nocodazole for 19 hours. Therefore, we showed that once cells are in mitosis, zinc is no longer a requirement. However, following supplementation with zinc after nocodazole treatment, no phosphorylation of Histone H3(Ser10) was seen and levels of pSTAT3(Ser727) were decreased, indicative of non-mitotic cells (*Figure 11*). This seems to contradict the suggestion that zinc drives mitosis. An alternative interpretation is that the cells have already progressed through mitosis, which is supported by fluorescent microscopy analysis. In TamR cells, treated in the same way with nocodazole for 20 hours with the addition of 100μM zinc for the last 15, 30, 45 and 60 minutes, phosphorylation of Histone H3(Ser10) was seen as well as a reduction in mitosis, indicative of cells in the latter stages of cell division (*Figure 15*) (Taylor, unpublished). The cells seemed to have progressed through mitosis so that p-Histone

H3(Ser10) had become dephosphorylated, accounting for the lack of expression in zinc-treated cells. This suggests that zinc treatment causes the cell to continue through mitosis, overriding the microtubule-interfering properties of nocodazole.

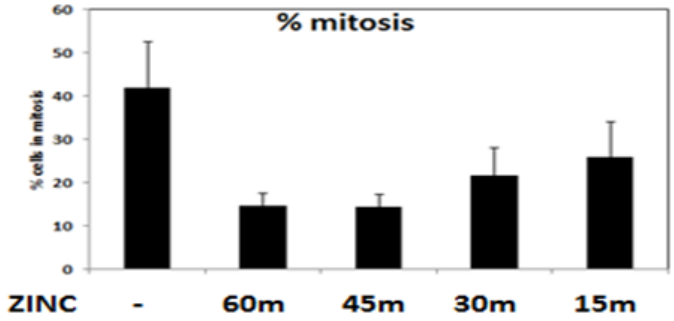

Figure 15: TamR cells were treated with 100ng/ml nocodazole for 20 hours, with addition of 100μM zinc for 0-60 minutes as indicated. Zinc treatment shows decreased percentage of mitotic cells (adopted from Taylor, unpublished).

An interesting observation was also made, that the full length STAT3 band disappeared at the end of mitosis (*Figure 11*). This suggests STAT3 is C-terminally cleaved at the end of mitosis which would result in the removal of Serine727, thus, preventing phosphorylation of this residue and only allowing phosphorylation of Tyrosine705.
This means that zinc treatment of mitotic TamR cells results in the proteolytic cleavage of STAT3, presumably by C-terminal cleavage, which provides a possible mechanism whereby pSTAT3(Ser727) is deactivated at the end of mitosis.

A further backup mechanism could involve the activity of protein phosphatases, as zinc in the presence of the zinc ionophore pyrithione significantly decreased protein phosphatase 2A (PP2A) activity (Lee et al. 2009). PP2A is responsible for STAT3 serine dephosphorylation (Liang et al. 1999). Therefore, it is possible that a reduction in zinc at the end of mitosis may activate PP2A leading to dephosphorylation of pSTAT3(Ser727). This suggestion is supported by the finding that PP2A dephosphorylates stathmin. Unphosphorylated stathmin is reported to destabilize microtubules while phosphorylated stathmin stabilizes them during mitosis (Tournebize et al. 1997). It is therefore suggested, in this study, that phosphorylated stathmin binds pSTAT3(Ser727) during mitosis and PP2A is responsible for the dephosphorylation of stathmin at the end of mitosis. PP2A may also cause the dephosphorylation of pSTAT3(Ser727) at the end of mitosis.

8. Conclusion

In summary, this research provides evidence for the involvement of both pSTAT3(Ser727) and zinc transporter ZIP6 in mitosis. The implications of this study are large. The interplay between ZIP6 and pSTAT3(Ser727) cites zinc as an intracellular signalling potential initiator, which leads to cell division. Due to its ubiquitous vital role in cells, zinc itself does not represent a potential therapeutic target. Our findings coupled with a previous report that STAT3-deficient mice die during early embryogenesis (Akira, 1999) provide evidence that STAT3 is crucial for mitosis and may be a suitable potential target for inhibiting cell proliferation. This knowledge may also be extended to other cancer cell lines and diseases where overactive cell proliferation is a common feature. In terms of future directions, investigation of the mechanism by which pSTAT3(Ser727) is deactivated at the end of mitosis either through the activity of PP2A in mitosis or proteolytic cleavage, remains to be researched. The recent identification of CK2 as the kinase responsible for ZIP7 activation which regulates the release of zinc from intracellular stores (Taylor et al. 2012), suggesting that there may be a protease followed by a kinase responsible for the activation and cleavage of ZIP6, may present a new and novel therapeutic target for breast cancer.

9. References

Akira S. Functional roles of STAT family proteins: lessons from knockout mice.Stem Cells. 1999;17(3):138-46

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, W: Garland Sciencealter P. Molecular biology of the cell. 2002. New York

American Cancer Society. Breast Cancer, Facts and Figures 2011-2012, [online] (accessed 2013-03-31), <http://www.cancer.org/acs/groups/content/@epidemiologysurveilance/documents/document/acspc-030975.pdf.>

Andreini C, Banci L, Bertini I, Rosato A. Counting the zinc-proteins encoded in the human genome. J Proteome Res. 2006 Jan;5(1):196-201

Antoniou AC, Pharoah PDP, Smith P, Easton DF. The BOADICEA model of genetic susceptibility to breast and ovarian cancer.Br. J. Cancer. 2004;91:1580–1590

Beatson GT. On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. Lancet 2, 1896, 105-107

Berger CE, Qian Y, Liu G, Chen H, Chen X. p53, a target of estrogen receptor (ER) α, modulates DNA damage-induced growth suppresion in ER-positive breast cancer cells.J Biol Chem. 2012 Aug 31;287(36):30117-27. doi: 10.1074/jbc.M112.367326. Epub 2012 Jul 11

Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. Oncogene. 2000 May 15;19(21):2474-88

Boyd NF, Martin LJ, Yaffe MJ, Minkin S. Mammographic density and breast cancer risk: current understanding and future prospects. Breast Cancer Research.2011;13(6): 223

Breastcancer.org. DES(diethylstilbestrol) Exposure, 2013, [online] (accessed 2013-04-07), <http://www.breastcancer.org/risk/factors/des_exposure>

Breastcancer.org. Exposure to Chemicals in Cosmetics, 2013, [online] (accessed 2013-04-07), <http://www.breastcancer.org/risk/factors/cosmetics>

Breastcancer.org, Menstrual History, 2013, [online] (accessed 2013-04-01), <http://www.breastcancer.org/risk/factors/menstrual_hist>

Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. Clin Cancer Res. 2002 Apr;8(4):945-54

Cahyadi S. Zinc in the Retinal Pigment Epithelium and Choriocapillaris Interface. PhD Thesis, University College London, 2011

Cancer research UK. Breast cancer risk factors, 2009, [online] (accessed 2013-04-01),

<http://www.cancerresearchuk.org/cancer-info/cancerstats/types/breast/riskfactors/.>

Cancer research UK. Types of breast cancer hormone therapy, 2013, [online] (accessed 2013-06-01) <http://www.cancerresearchuk.org/cancer-help/type/breast-cancer/treatment/hormone/types-of-breast-cancer-hormone-therapy>

Casey G. (1997). "The BRCA1 and BRCA2 breast cancer genes."Curr Opin Oncol.1997 Jan;9(1):88-93

Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R, Ciliberto G, Moscinski L, Fernandez-Luna JL, Nunez G, Dalton WS, Jove R. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity. 1999 Jan;10(1):105-15

Cauley JA, Gutai JP, Kuller LH, LeDonne D, Powell JG. The epidemiology of serum sex hormones in postmenopausal women, Am J Epidemiol. 1989 Jun;129(6):1120-31

Cavallo F, Gerber M, Marubini E, Richardson S, Barbieri A, Costa A, DeCarli A, Pujol H. Zinc and copper in breast cancer. A joint study in northern Italy and southern France.Cancer. 1991 Feb 1;67(3):738-45

Chesters JK, Petrie L.A possible role for cyclins in the zinc requirements during G1 and G2 phases of the cell cycle.J Nutr Biochem. 1999 May;10(5):279-90

Chung J, Uchida E, Grammer TC, Blenis J. STAT3 serine phosphorylation by ERK-dependent and –independent pathways negatively modulates its tyrosine phosphorylation. Mol Cell Biol. 1997 Nov;17(11):6508-16

Claus EB, Risch N,Thompson WD. Genetic analysis of breast cancer in the cancer and steroid hormone study. Am J Hum Genet. 1991 Feb;48(2):232-42

Coleman MP, Est'eve J, Damiecki P, Arslan A, Renard H. Trends in Cancer Incidence and Mortality, IARC Sci Publ. 1993;(121):1-806

Collaborative Group on Hormonal Factors in Breast Cancer, Breast cancer and hormonal contraceptives: collaborative reanalysis of individual data on 53 297 women with breast cancer and 100 239 women without breast cancer from 54 epidemiological studies. Collaborative Group on Hormonal Factors in Breast Cancer.Lancet. 1996 Jun 22;347(9017):1713-27

Collaborative Group on Hormonal Factors in Breast Cancer, Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Lancet. 1997 Oct 11;350(9084):1047-59

Collaborative Group on Hormonal Factors in Breast Cancer, Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50302 women with breast cancer and 96973 women without disease. Lancet. 2002 Jul 20;360(9328):187-95

Collaborative Group on Hormonal Factors in Breast Cancer. 2001. Familial breast cancer: collaborative reanalysis of individual data from 52 epidemiological studies including 58.209 women with breast cancer and 101.986 women without the disease. Lancet;358(9291):1389-1399

Crew KD. Vitamin D: are we ready to supplement for breast cancer prevention and treatment? ISRN Oncol. 2013;2013:483687. doi: 10.1155/2013/483687. Epub 2013 Feb 26

Darbre PD. Underarm cosmetics and breast cancer.J Appl Toxicol. 2003 Mar-Apr;23(2):89-95

Dauer DJ, Ferraro B, Song L, Yu B, Mora L, Enkemann S, Jove R, Haura EB. Stat3 regulates genes common to both wound healing and cancer. Oncogene. 2005 May 12;24(21):3397-408

Decker T, Kovarik P. Serine phosphorylation of STATs.Oncogene. 2000 May 15;19(21):2628-37

Easton DF, Ford D, Bishop DT. Breast and ovarian cancer incidence in BRCA1-mutation carriers.Breast Cancer Linkage Consortium.Am J Hum Genet.1995 Jan;56(1):265-71

Ehsani S, Salehzadeh A, Huo H, Reginold W, Pocanschi CL, Ren H, Wang H, So K, Mehrabian M, Strome R, Trimble WS, Hazrati LN, Rogaeva E, Westaway D, Carlson GA, Schmitt-Ulms G. LIV-1 ZIP ectodomain shedding in prion-infected mice resembles cellular response to transition metal starvation. J Mol Biol. 2012 Sep 28;422(4):556-74. doi: 10.1016/j.jmb.2012.06.003. Epub 2012 Jun 9

Eide DJ. Zinc transporters and the cellular trafficking of zinc. Biochim Biophys Acta. 2006 Jul;1763(7):711-22. Epub 2006 Apr 18

Fisher B, Dignam J, Bryant J, Wolmark N. Five versus more than five years of tamoxifen therapy for breast cancer patients with negative lymph nodes and estrogen receptor-positive tumors. J Natl Cancer Inst. 1996;88:1529–1542

Fujioka M, Lieberman I. A Zn++ requirement for synthesis of deoxyribonucleic acid by rat liver.J Biol Chem. 1964 Apr;239:1164-7

Gaither LA, Eide DJ. Eukaryotic zinc transporters and their regulation.Biometals. 2001 Sep-Dec;14(3-4):251-70

Garcia R, Bowman TL, Niu G, Yu H, Minton S, Muro-Cacho CA, Cox CE, Falcone R, Fairclough R, Parsons S, Laudano A, Gazit A, Levitzki A, Kraker A, Jove R. Constitutive activation of Stat3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. Oncogene. 2001 May 3;20(20):2499-513

Garcia R, Yu CL, Hudnall A, Carlett R, Nelson KL, Smithgall T, Fujita DJ, Ethier SP, Jove R. Constitutive activation of Stat3 in fibroblasts transformed by diverse oncoproteins and in breast carcinoma cells. Cell Growth Differ. 1997 Dec;8(12):1267-76

Guerinot ML. The ZIP family of metal transporters.Biochim Biophys Acta.2000 May 1;1465(1-2):190-8

Gupta SK, Shukla VK, Vaidya MP, Roy SK, Gupta S. Serum trace elements and Cu/Zn ration in breast cancer patients. J Surg Oncol. 1991 Mar;46(3):178-81

Haase H, Maret W. Intracellular zinc fluctuations modulate protein tyrosine phosphatase activity in insulin/insulin-like growth factor-1 signaling. Exp Cell Res. 2003 Dec 10;291(2):289-98

Hansson A. Extracellular zinc ions induces mitogen-activated protein kinase activity and protein tyrosine phosphorylation in bombesin-sensitive Swiss 3T3 fibroblasts. Arch Biochem Biophys. 1996 Apr 15;328(2):233-8

Hevehan DL, Miller WM, Papoutsakis ET. Differential expression and phosphorylation of distnict STAT3 proteins during granulotyc differentiation.Blood. 2002 Mar 1;99(5):1627-37

Hildreth NG, Shore RE, Dvoretsky PM. The risk of breast cancer after irradiation of the thymus in infancy, New Engl. J. Med., 321 (1989), pp. 1281–1284

Hiscox S, Morgan L, Barrow D, Dutkowski C, Wakeling A, Nicholson RI. Tamoxifen resistance in breast cancer cells is accompanied by an enhanced motile and invasive phenotype: Inhibition by gefitinib (´Iressa´, ZD1839). Clinical and Experimental Metastasis. 2004:21(3):201-212

Huggins Ch, Endocrine-induced regression of cancers, Nobel Lecture, December 13, 1966, [online] (accessed 2013-06-01), <http://www.nobelprize.org/nobel_prizes/medicine/laureates/1966/huggins-lecture.pdf.>

Hulka BS, Moorman PG. Breast cancer: hormones and other risk factors. Maturitas. 2001 Feb 28;38(1):103-13; discussion 113-6

Jaiyesimi IA, Buzdar AU, Decker DA, Hortobagyi GN. Use of tamoxifen for breast cancer: twenty-eight years later. J Clin Oncol.1995;13:513–529

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. 2011. Global cancer statistics, CA: A Cancer Journal for Clinicians, Mar-Apr;61(2):69-90. Epub 2011 Feb 4
Kamangar F, Dores G, Anderson W. Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. J Clin Oncol. 2006 May 10;24(14):2137-2150

Kambe T, Andrews GK. Novel proteolytic processing of the ectodomain of the zinc transporter ZIP4 (SLC39A4) during zinc deficiency is inhibited by acrodermatitis enterohepathica mutations. Mol Cell Biol. 2009 Jan;29(1):129-39. doi: 10.1128/MCB.00963-08. Epub 2008 Oct 20

Kambe T. An overview of a wide range of functions of ZnT and Zip zinc transporters in the secretory pathway. Biosci Biotechnol Biochem. 2011;75(6):1036-43. Epub 2011 Jun 13

Kato T, Sakamoto E, Kutsuna H, Kimura-Eto A, Hato F, Kitagawa S. Proteolytic conversion of STAT3alpha to STAT3gamma in human neutrophils: role of granule-derived serine proteases. J Biol Chem. 2004 Jul 23;279(30):31076-80. Epub 2004 May 15

Kelsey JL, Gammon MD, John EM. Reproductive factors and breast cancer.Epidemiol Rev.1993;15(1):36-47

Key J, Hodgson S, Omar RZ, et al. Meta-analysis of studies of alcohol and breast cancer with consideration of the methodological issues.Cancer Causes Control. 2006;17:759-770

Key TJ, Pike MC. The role of oestrogens and progestagens in the epidemiology and preventiv of breast cancer, Eur J Cancer Clin Oncol. 1988 Jan;24(1):29-43

Key TJ, Verkasalo PK, Banks E. Epidemiology of breast cancer, Lancet Oncol. 2001 Mar;2(3):133-40

Kitabayashi C, Fukada T, Kanamoto M, Ohashi W, Hojyo S, Atsumi T, Ueda N, Azuma I, Murakami M, Hirano T. Zinc suppresses Th17 development via inhibition of STAT3 activation. IntImmunol. 2010 May;22(5):375-86. doi: 10.1093/intimm/dxq017. Epub 2010 Mar 9

Knowlden JM, Hutcheson IR, Jones HE, Madden T, Gee JM, Harper ME, Barrow D, Wakeling AE, Nicholson RI. Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. Endocrinology. 2003 Mar;144(3):1032-44

Kuller LH, Cauley JA, Lucas L, Cummings S, Browner WS. Sex steroid hormones, bone mineral density, and risk of breast cancer.Environ Health Perspect. 1997 Apr;105 Suppl 3:593-9

Lee S, Chanoit G, McIntosh, Zvara DA, Xu Z. Molecular mechanism underlying Akt activation in zinc-induced cardioprotection. Am J Physiol Heart Circ Physiol. 2009 Aug;297(2):H569-75. doi: 10.1152/ajpheart.00293.2009. Epub 2009 Jun 12

Leonardi GC, Rapisarda V, Marconi A, Scalisi A, Catalano F, Proietti L, Travali S, Libra M, Fenga C. Correlation of the risk of breast cancer and disruption of the circadian rhythm. Oncol Rep. 2012 Aug;28(2):418-28. doi: 10.3892/or.2012.1839. Epub 2012 May 29

Li Y, Maret W. Transient fluctuations of intracellular zinc ions in cell proliferation. Exp Cell Res. 2009 Aug 15;315(14):2463-70. doi: 10.1016/j.yexcr.2009.05.016. Epub 2009 May 23

Liang H, Venema VJ, Wang X, Ju H, Venema RC, Marrero MB. Regulation of angiotensin II-induced phosphorylation of STAT3 in vascular smooth muscle cells.J Biol Chem. 1999 Jul 9;274(28):19846-51

Liuzzi JP, Cousins RJ. Mammalian zinc transporters.Annu Rev Nutr. 2004;24:151-72

Ma H, Bernstein L, Pike MC, Ursin G. Reproductive factors and breast cancer risk according to joint estrogen and progesterone receptor status: a meta-analysis of epidemiological studies. Breast Cancer Res. 2006 ;8(4):R43

MacMahon B, Cole P, Lin TM, Lowe CR, Mirra AP, Ravnihar B, et al. Age at first birth and breast cancer risk.Bull World Health Organ. 1970;43(2):209–221

Manning DL, McClelland RA, Gee JM, Chan CM, Green CD, Blamey RW, Nicholson RI. The role of fouroestrogen-responsivegenes, pLIV1, pS2, pSYD3 and pSYD8, in predicting responsiveness to endocrine therapy in primary breast cancer.Eur J Cancer. 1993;29A(10):1462-8

Manning DL, McClelland RA, Knowlden JM, Bryant S, Gee JM, Green CD, Robertson JF, Blamey RW, Sutherland RL, Ormandy CJ, et al. Differential expression of oestrogen regulated genes in breast cancer. Acta Oncol. 1995;34(5):641-6

Margalioth EJ, Schenker JG, Chevion M. Copper and zinc levels in normal and malignant tissues. Cancer. 1983 Sep 1;52(5):868-72

McCormack VA, dos Santos Silva I. Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis. Cancer Epidemiol Biomarkers Prev. 2006 Jun;15(6):1159-69

MCF-7 Cells, human breast adenocarcinoma cell line, General Information, [online] (accessed 2013-03-18), <http://mcf7.com/>

Memon AU, Kazi TG, Afridi HI, Jamali MK, Arain MB, Jalbani N, Syed N. Evaluation of zinc status in whole blood and scalp hair of female cancer patients. Clin Chim Acta. 2007 Apr;379(1-2):66-70. Epub 2006 Dec 22

Miki Y, Swensen J, Shattuck-Eidens D, et al: A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 266: 66-71, 1994

Miller AB, Howe GR, Sherman GJ, Lindsay JP, Jaffe MJ, Dinner PJ et al. Mortality from breast cancer after irradiation during fluoroscopic examinations in patients being treated for tuberculosis, New Engl. J. Med., 321 (1989), pp. 1285–1289

Mulay IL, Roy R, Knox BE, Suhr NH, Delaney WE. Trace-metal analysis of cancerous and noncancerous human tissues.J Natl Cancer Inst. 1971 Jul;47(1):1-13

Murgia C, Vespignani I, Cerase J, Nobili F, Perozzi G. Cloning, expression, and vesicular localization of zinc transporter Dri27/ZnT4 in intestinal tissue and cells. Am J Physiol. 1999 Dec;277(6 Pt 1):G1231-9

Ng J, Cantrell D. STAT3 is a serine kinase target in T lymphocytes. Interleukin 2 and T cell antigen receptor signals converge upon serine 727. J Biol Chem.1997 Sep 26;272(39):24542-9

Nichols M. The fight against tamoxifen resistence in breast cancer therapy?A new target in the battle?Mol Interv.2007 Feb;7(1):13-6

Palmer JR, Rosenberg L, Clarke EA, Stolley PD, Warshauer ME, Zauber AG, Shapiro S. Breast cancer and cigarette smoking: a hypothesis. Am J Epidemiol. 1991 Jul 1;134(1):1-13

Palmiter RD, Findley SD. Cloning and functional characterization of a mammalian zinc transportet that confers resistance to zinc. EMBO J. 1995 Feb 15;14(4):639-49

Palmiter RD, Huang L. Efflux and compartmentalization of zinc by members of the SLC30 family of solute carriers. Pflugers Arch. 2004 Feb;447(5):744-51. Epub 2003 May 14

Parkin DM, Whelan SL, Ferlay J, Raymond L, Young J (Eds.). Cancer Incidence in Five Continents, International Agency for Research on Cancer, Oxford University Press, Oxford (1997)

Piccinini L, Borella P, Bargellini A, Medici CI, Zoboli A. A case-control study on selenium, zinc, and copper in plasma and hair of subjects affected by breast and lung cancer.Biol Trace Elem Res. 1996 Jan;51(1):23-30

Ranger JJ, Levy DE, Shahalizadeh S, Hallett M, Muller WJ.Identification of a Stat3-dependent transcription regulatory network involved in metastatic progression.Cancer Res. 2009 Sep 1;69(17):6823-30. doi: 10.1158/0008-5472.CAN-09-1684. Epub 2009 Aug 18

Sainsbury R. Aromatase inhibition in the treatment of advanced breast cancer: is there a relationship between potency and clinical efficacy? Br J Cancer.2004 May 4; 90(9): 1733–1739.

Santoliquido PM, Southwick HW, Olwin JH. Trace metal levels in cancer of the breast. Surg Gynecol Obstet. 1976 Jan;142(1):65-70

Sato K, Sato A, Aoto M, Fukami Y. C-Src phosphorylates epidermal growth factor receptor on tyrosine 845. Biochem Biophys Res Commun. 1995 Oct 24;215(3):1078-87

Schaefer TS, Sanders LK, Park OK, Nathans D. Functional differences between STAT3alpha and Stat3beta.Mol Cell Biol. 1997 Sep;17(9):5307-16

Schatzkin A, Longnecker MP. Alcohol and breast cancer. Where are we now and where do we go from here? Cancer. 1994 Aug 1;74(3 Suppl):1101-10

Schippinger W, Ploner F, Poustka A, Sultmann H. Identification and meta-analysis of a small gene expression signature for the diagnosis of estrogen receptor status in invasive ductal breast cancer. Int J Cancer. 2006 Dec 15;119(12):2974-9

Schmitt-Ulms G, Ehsani S, Watts JC, Westaway D, Wille H. Evolutionary descent of prion genes from the ZIP family of metal ion transporters. PLoS One. 2009 Sep 28;4(9):e7208. doi: 10.1371/journal.pone.0007208

Schneider J, Ruschhaupt M, Buness A, Asslaber M, Regitnig P, Zatloukal K, Schubert EL, Mefford HC, Dann JL, Argonza RH, Hull J, King MC. BRCA1 and BRCA2 mutations in Ashkenazi Jewish families with breast and ovarian cancer. Genet Test. 1997;1(1):41-6

Schneider J, Ruschhaupt M, Buness A, Asslaber M, Regitnig P, Zatloukal K, Schippinger W, Ploner F, Poustka A, Sultmann H. Identification and meta-analysis of a small gene expression signature for the diagnosis of estrogen receptor status in invasive ductal breast cancer. Int J Cancer. 2006 Dec 15;119(12):2974-9

Schubert EL, Mefford HC, Dann JL, Argonza RH, Hull J, King MC: BRCA1 and BRCA2 mutations in Ashkenazi Jewish families with breast and ovarian cancer. Genet Test. 1997;1(1):41-6.

Seve M, Chimienti F, Devergnas S, Favier A. In silico identification and expression of SLC30 family genes: an expressed sequence tag data mining strategy for the characterization of zinc transporterś tissue expression. BMC Genomics. 2004 May 23;5(1):32

Shi X, Zhang H, Paddon H, Lee G, Pelech S. Phosphorylation of STAT3 serine-727 by cyclin-dependent kinase 1 is critical for nocodazole-induced mitotic arrest. Biochemistry. 2006 May 9;45(18):5857-67

Shih HA, Couch FJ, Nathanson KL, Blackwood MA, Rebbeck TR, Armstrong KA, Calzone K, Stopfer J, Seal S, Stratton MR, Weber BL. BRCA1 and BRCA2 mutation frequency in women evaluated in a breast cancer risk evaluation clinic.J Clin Oncol. 2002 Feb 15;20(4):994-9

Shuai K, Horvath CM, Huang LH, Qureshi SA, Cowburn D, Darnell JE Jr. Interferon activation of the transcription factor Stat91 involves dimerization through SH2-phosphotyrosyl peptide interactions. Cell. 1994 Mar 11;76(5):821-8

Siegel R, Naishadham D, Jemal A, Cancer statistics, 2012. In: CA: A Cancer Journal for Clinicians, Volume 62, Issue 1, 2012:10-29

Smith P, McGuffog L, Easton DF, Mann GJ, Pupo GM, Newman B, Chenevix-Trench G; kConFab Investigators, Szabo C, Southey M, Renard H, Odefrey F, Lynch H, Stoppa-Lyonnet D, Couch F, Hopper JL, Giles GG, McCredie MR, Buys S, Andrulis I, Senie R; BCFS, BRCAX Collaborators Group, Goldgar DE, Oldenburg R,Kroeze-Jansema K, Kraan J, Meijers-Heijboer H, Klijn JG, van Asperen C, van Leeuwen I, Vasen HF, Cornelisse CJ, Devilee P, Baskcomb L, Seal S, Barfoot R,Mangion J, Hall A, Edkins S, Rapley E, Wooster R, Chang-Claude J, Eccles D, Evans DG, Futreal P, Nathanson KL, Weber BL; Breast Cancer SusceptibilityCollaboration (UK), Rahman N, Stratton MR. A genome wide linkage search for breast cancer susceptibility genes. Genes Chromosom. Cancer.2006;45:646–655

Soule HD; Vazquez J, Long A, Albert S, Brennan M. (1973). "A human cell line from a pleural effusion derived from a breast carcinoma". Journal of the National Cancer Institute51 (5): 1409–1416

Taylor KM, Hiscox S, Nicholson RI, Hogstrand C, Kille P. Protein kinase CK2 triggers cytosololic zinc signaling pathways by phosphorylation of zinc channel ZIP7. Sci Signal. 2012 Feb 7;5(210):ra11. doi: 10.1126/scisignal.2002585

Taylor KM, Hiscox S, Nicholson RI. Zinc transporter LIV-1: a link between cellular development and cancer progression. Trends Endocrinol Metab. 2004 Dec;15(10):461-3

Taylor KM, Nicholson RI. The LZT proteins, the LIV-1 subfamily of zinc tranposters. Biochim Biophys Acta. 2003 Apr 1;1611(1-2):16-30

Taylor KM, Vichova P, Jordan N, Hiscox S, Hendley R, Nicholson RI. ZIP7-mediated intracellular zinc transport contributes to aberrant growth factor signaling in antihormone-resistant breast cancer cells. Endocrinology. 2008 Oct;149(10):4912-20. doi: 10.1210/en.2008-0351. Epub 2008 Jun 26

Taylor KM, Morgan HE, Johnson A,Hadley LJ, Nicholson RI. Structure-Function Analysis of LIV-1, The breast cancer-associated protein that belongs to a new subfamily of zinc transporters. Biochemical Journal. 2003;375:51-59

the Jena Centre for Bioinformatics, Bioinformatics, SDS-PAGE, [online] (accessed 2013-03-25),

<http://www.imb-jena.de/~rake/Bioinformatics_WEB/proteins_purification.html>

Thermo Scientific Pierce Protein Biology products, Overview of Western Blotting, [online] (accessed 2013-03-26),
<http://www.piercenet.com/browse.cfm?fldID=8259A7B6-7DA6-41CF-9D55-AA6C14F31193>

Tournebize R, Andersen SS, Verde F, Dorée M, Karsenti E, Hyman AA. Distinct roles of PP1 and PP2A-like phosphatases in control of microtubule dynamics during mitosis. EMBO J. 1997 Sep 15;16(18):5537-49

Trichopoulos D, MacMahon B, Cole P. Menopause and breast cancer risk, J Natl Cancer Inst. 1972 Mar;48(3):605-13

Vallee BL, Falchuk KH. The biochemical basis of zinc physiology.Physiol Rev. 1993 Jan;73(1):79-118

Wakahara R, Kunimoto H, Tanino K, Kojima H, Shintaku H, Nakajima K. Phospho-Ser727 of STAT3 regulates STAT3 activity by enhancing dephosphorylation of phospho-Tyr705 largely through TC45. Genes Cells. 2012 Feb;17(2):132-45. doi: 10.1111/j.1365-2443.2011.01575.x. Epub 2012 Jan 11

Wen Z, Zhong Z, Darnell JE Jr. Maximal activation of transcriptional by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. Cell. 1995 Jul 28;82(2):241-50

Woo W, Xu Z. Body zinc distribution profile during N-methyl-N-nitrosourea-induced mammary tumorigenesis in rats at various levels of dietary zinc intake. Biol Trace Elem Res. 2002 Summer;87(1-3):157-69

Wooster R, Bignell G, Lancaster J, et al. Identification of the breast cancer susceptibility gene BRCA2.Nature378: 789-792, 1995

Wu W, Graves LM, Jaspers I, Devlin RB, Reed W, Samet JM. Activation of the EGF receptor signaling pathway in human airway epithelial cells exposed to metals. Am J Physiol. 1999 Nov;277(5 Pt 1):L924-31

Yamashita S, Miyagi C, Fukada T, Kagara N, Che YS, Hirano T. Zinc transporter LIV1 controls epithelial-mesenchymal transition in zebrafish gastrula organizer. Nature. 2004 May 20;429(6989):298-302. Epub 2004 May 5

Yucel I, Arpaci F, Ozet A, Doner B, Karayilanoglu T, Sayar A, Berk O. Serum copper and zinc levels and copper/zinc ratio in patients with breast cancer. Biol Trace Elem Res. 1994 Jan;40(1):31-8

Zalewski P. Zinc in Mammalian Cell Cycle and Cell Death. In: Rink L. Ed. Zinc in Human Health. Amsterdam: IOS Press BV, pp. 63-93

Zhang X, Blenis J, Li HC, Schindler C, Chen-Kiang S. Requirement of serine phosphorylation for formation of STAT-promoter complexes.Science. 1995 Mar 31;267(5206):1990-4

Zhong Z, Wen Z, Darnell JE Jr. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. Science. 1994 Apr 1;264(5155):95-8